1994

Electrophysiological Responses of Olfactory Receptor and Bulb Neurons to Stimulus Mixtures and to Their Individual Components in the Channel Catfish, Ictalurus Punctatus.

Jiesheng Kang
Louisiana State University and Agricultural & Mechanical College

Order Number 9508576

Electrophysiological responses of olfactory receptor and bulb neurons to stimulus mixtures and to their individual components in the channel catfish, *Ictalurus punctatus*

Kang, Jiesheng, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1994

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

ELECTROPHYSIOLOGICAL RESPONSES OF OLFACTORY RECEPTOR AND BULB NEURONS TO STIMULUS MIXTURES AND TO THEIR INDIVIDUAL COMPONENTS IN THE CHANNEL CATFISH, *ICTALURUS PUNCTATUS*

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Zoology and Physiology

by
Jiesheng Kang
B.S., Xiamen University, China, 1982
M.S., Louisiana State University, 1990
August 1994

ACKNOWLEDGEMENTS

Sincerest thanks to my major advisor, Dr. John Caprio, for the opportunity, the direction, and the support he has provided me in this endeavor. This thesis would not have been possible without his guidance on issues relating to as well as extending beyond the realm of science. I am also grateful to Dr. Kathryn Hamilton for her inspiration and patience during my visits to her laboratory, her expert technical assistance with the antidromic experiments included in this dissertation and her critical review of the manuscript. My gratitude extends to all of the remaining members of my committee, Drs. Thomas Dietz, Michael Fitzsimons, Peter Johnsen and George Strain for serving on my graduate committee; I thank them for their encouragement and inspiration during this endeavor and reviewing the dissertation. Others deserving acknowledgement include my colleagues Dr. Charles Lamb, Dr. Jun Kohbara and Ms. Sandra Wegert for their kind help during the initial stages of this project. I also thank Dr. Rainer Voigt of Boston University Marine Program, Woods Hole, MA, for BASIC-language programs that aided our computer analysis of the data, Mr. Ron Bouchard for his time and effort in the darkroom, Dr. David Blouin for introducing the statistical programs (interrupted time-series analysis and MANOVA), and Dr. Thomas Finger for the opportunity he provided for me to learn basic histological techniques in his laboratory. This project was supported by ONR grant N00014-90-J-1583 to Dr. John Caprio.

Finally, I thank my wife, Jie Zhang, for her support and patience throughout the process, and my mother, Swan Zhang, for her understanding, wisdom and love.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (*con'd.*)

ABSTRACT

Both electro-olfactogram and integrated multiunit receptor responses of populations of olfactory receptor neurons (ORNs) clearly showed that responses to complex mixtures were predictable. Results demonstrated that receptor sites for basic and acidic amino acids, respectively, are independent from those for neutral amino acids and multiple receptor site types with highly overlapping specificities exist for amino acids within each group. Results also confirmed that one mechanism for synergism is the simultaneous activation of relatively independent receptor sites by the components in a mixture, and there was no evidence of mixture suppression.

Single ORNs recorded *in vivo* responded to stimuli with either excitation, suppression or both. Results suggested that suppressive responses, as well as excitatory responses, play important roles in olfactory coding. The result that 55% of the single ORNs studied responded to more than one stimulus suggested that multiple receptor site types for different odorants were expressed in the plasma membranes of single ORNs. Responses of single ORNs to binary mixtures of amino acids indicated that although mixture interactions occurred, none changed response types from those observed to the individual components. Thus, the observed response types to the tested component-similar binary mixtures were predictable.

Responses of single olfactory bulb neurons to amino acids were highly reproducible over time. Results suggested that suppressive as well as excitatory responses are involved in both quality and quantity coding of olfactory information. Response types of olfactory bulb

neurons to a given stimulus at different concentrations did not change from excitation to suppression, or *vice versa*.

The responses of single olfactory bulb neurons to binary mixtures were highly associated with the responses to the components. Although mixture interactions occurred, mixture interactions that changed olfactory bulb neuron response types from those observed to the individual components were rare. The results indicated that responses to binary mixtures whose components evoked the same response types were generally predictable, whereas predictability of responses to the component-different binary mixtures was dependent upon the specific types of mixtures tested.

INTRODUCTION

Physiological investigations of vertebrate olfaction have classically studied how the olfactory system responds to single odorants. Under natural circumstances, however, olfactory receptor neurons (ORNs) encounter a complex mixture of odorants varying in concentration and potency. Thus, studying the ways in which stimulus mixtures interact with olfactory receptor neurons is critical for an understanding of olfactory coding, i.e., how action potentials generated by ORNs in response to odorants code the stimulus molecule(s). That an intact olfactory organ is necessary for the discrimination of odorants by catfish was recently shown (Valentincic et al., 1994). From electrophysiological, biochemical and molecular studies, two main hypotheses were proposed as to how olfactory receptor neurons code odorant quality. The "labeled line" coding scheme assumes that for each individual odor quality, there must be a population of olfactory receptor neurons that codes this quality exclusively (Lancet, 1991; Kauer, 1991; Derby and Atema, 1988; De Jong and Visser, 1988; Derby and Ache, 1984a). Furthermore, the population of receptor neurons must have a similar, but "narrowly-tuned" response spectrum, which is different from that of other populations of receptor neurons. The primary alternative coding scheme, "across-fiber" patterning, assumes that one odor quality is coded by the activity pattern elicited by the stimulus across a population of neurons (Scholz et al., 1993; Kauer, 1991; Derby and Ache, 1984a; Derby et al., 1984; De Jong and Visser, 1988; Carr and Derby, 1986b).

A key difference between these two coding schemes depends upon the distribution of receptor molecules across the entire population of olfactory receptor neurons. Thus, by comparing the responses of olfactory receptor neurons to stimulus mixtures and to their components, it is possible to address whether there are different receptor site types for different amino acids, and if they are different, how are they distributed across single olfactory receptor neurons.

Whether the olfactory system processes information concerning stimulus mixtures differently than how it processes information concerning individual odorants is a critical issue in olfaction. Numerous studies, especially those involving crustaceans, reported that because of mixture interactions, mixture suppression and synergism, olfactory receptor responses to stimulus mixtures were generally unpredictable (Atema et al., 1989; Derby and Ache, 1984b; Zimmer-Faust et al., 1984; Derby et al., 1985; Gleeson and Ache, 1985; Johnson et al., 1985; Johnson et al., 1989; Borroni et al., 1986; Carr and Derby, 1986a; Carr and Derby, 1986b; Derby et al., 1991a; Derby et al., 1991b). Mixture suppression occurs if the measured responses are significantly less than the predicted responses, whereas synergism results if the measured responses are significantly greater than predicted. Thus, the general conclusion of these previous studies is that chemical mixtures are treated differently by the olfactory system than their individual components. This conclusion implies that possibly a new coding principle, different from that utilized for single odorants is used when ORNs simultaneously detect multiple

odorants. Thus, it is essential for a better understanding of the olfactory coding process that basic principles underlying how ORNs respond to odorant mixtures be investigated.

How neural information about odorants generated by ORNs is processed by neurons in the central olfactory system is yet another major issue for understanding olfactory coding. Neural processing in mitral cells, the output neurons of olfactory bulbs, is an important step in olfaction since the mitral cells receive input from ORNs and process and transmit neural information concerning odorants to higher levels within the central nervous system (CNS). The convergence ratio of vertebrate olfactory receptor neurons to mitral cells is estimated to be >1000:1 (Satou, 1990; Shepherd, 1979). Also, within the olfactory bulb numerous interneurons make synaptic connections with mitral cells and are involved in lateral inhibition (Satou, 1990; Shepherd, 1979). Further, centrifugal fibers make synaptic connections with granule cells and allow a centrifugal modulation. Because of these processes occurring within the olfactory bulb, whether mitral cells behave differently from ORNs in their responses to odorant mixtures, is critical for a better understanding of olfactory coding. The responses of single mitral cells to stimulus mixtures provide a window to the results of the neural processing of mixture information at the initial stage within the central nervous system.

In the present series of studies, electrophysiological responses of ORNs and olfactory bulb neurons were recorded *in vivo* in channel catfish, *Ictalurus punctatus*. Channel catfish is an excellent

experimental model for studying vertebrate olfaction since it possesses large olfactory organs which are accessible with minimal surgery for *in vivo* experiments, and is possesses ORNs that are highly sensitive to amino acids. Experimentally, these olfactory stimuli are presented naturally as aqueous solutions which allow stimulus concentrations to be easily manipulated. Finally much information exists concerning their olfactory system that was obtained from studies involving electrophysiology (Byrd and Caprio, 1982; Caprio, 1977; Caprio, 1978; Caprio, 1980; Caprio, 1988b; Caprio et al., 1989; Erickson and Caprio, 1984; Restrepo et al., 1990; Kang and Caprio, 1991; Miyamoto et al., 1992b; Miyamoto et al., 1992a; Ivanova and Caprio, 1993), biochemistry (Restrepo et al., 1993; Restrepo and Boyle, 1991; Bruch and Kalinoski, 1987; Kalinoski et al., 1987; Cancalon, 1978) and molecular biology (Ngai et al., 1993b; Ngai et al., 1993a; Goulding et al., 1992).

In the present study, chapter I was designed to investigate: (1) whether acidic, basic and neutral amino acids, respectively, bound to the same or relatively independent receptor sites, and (2) whether the basic principles that were successful in predicting electro-olfactogram (EOG) and integrated multiunit olfactory receptor responses to binary and trinary mixtures (Caprio et al., 1989) would be applicable to predicting the responses to more complex mixtures consisting of up to 10 different amino acids. The results indicated that: (a) acidic, basic, neutral with long side-chains and neutral with short side-chains amino acids, respectively, bind to a group of highly cross-reactive receptor site types, having highly overlapping

specificities, (b) the receptor site types for basic and acidic amino acids, respectively, are highly independent from those for neutral amino acids, and (c) olfactory receptor responses to complex mixtures of amino acids mostly follow the basic principles determined by studying simple stimulus mixtures and are experimentally predictable.

Chapter II was designed to investigate for the first time in any teleost: (1) the response specificity of single channel catfish ORNs recorded *in vivo*, (2) the relative occurrence of excitatory and suppressive responses, and (3) the responses of single ORNs to binary mixtures of amino acids. The results indicated that: (a) the majority of the single ORNs responded to more than one of the tested stimuli that were indicated to bind to different receptor sites, (b) single ORNs responded to the test stimuli with suppressive responses more frequently than with excitatory responses, and (c) no mixture interactions that changed response types from those observed to the individual components were encountered, indicating that the response types were predictable for binary mixtures whose components elicited the same response types.

Chapter III was designed to study the basic response properties of single olfactory bulb neurons to individual stimuli. This study was required for the subsequent investigation (Chapter IV) of the responses of olfactory bulb neurons to stimulus mixtures. The results indicated that: (a) responses of single olfactory bulb neurons to amino acids were highly reproducible over time, (b) responses of single olfactory bulb neurons to a given amino acid did not change from excitation to suppression, or *vice versa*, across different

stimulus concentrations, (c) the changes in magnitude of the responses of olfactory bulb neurons to increasing stimulus concentrations suggested that individual cells might discriminate stimulus intensity, and (d) with the sole exception of responses to L-methionine and L-norvaline, the overall responses to pairs of amino acids were not significantly correlated, indicating that the majority of amino acids tested were processed differently by the olfactory bulb.

Chapter IV was designed to study: (1) whether olfactory bulb neurons of the channel catfish respond to binary mixtures of amino acids differently than how they respond to the individual components tested separately, (2) whether mixture interactions are evident in the responses of olfactory bulb neurons to binary mixtures of amino acids, and (3) whether it is possible to predict the responses of single olfactory bulb neurons to binary mixtures based on the responses to the individual components. The results indicated that: (a) responses of single olfactory bulb neurons to binary mixtures were highly associated with the responses to the component amino acids, (b) mixture interactions that changed response types of olfactory bulb neurons from those observed to the individual components were rare, and (c) responses of single olfactory bulb neurons to binary mixtures whose components when tested individually resulted in the same response types were generally predictable; where response types to the individually tested components were different, the predictability of the responses was dependent upon the specific mixtures tested.

CHAPTER I

RESPONSES OF POPULATIONS OF OLFACTORY RECEPTORS TO COMPLEX MIXTURES OF AMINO ACIDS

INTRODUCTION

In nature, animals generally detect complex mixtures of odorants rather than single substances or simple mixtures that are usually presented to organisms in laboratory studies of olfaction. Whether stimulus mixtures are treated differently by olfactory receptors than individually presented stimuli is critical to a better understanding of the olfactory process. Two types of mixture interaction, mixture suppression and synergism, frequently have been cited as occurring in a number of studies in the chemical senses involving stimulus mixtures (Derby and Ache, 1984a; Bartoshuk and Gent, 1985; Johnson et al., 1989; Carr and Derby, 1986b; Carr and Derby, 1986a). Mixture suppression occurs if the measured responses are significantly less than the predicted response, often calculated by simply summing the responses to the individual components in the mixture. Synergism occurs if the measured responses are significantly greater than predicted. Possibly due to these mixture interactions, and in some cases to the experimental paradigm itself, numerous studies indicated the difficulty in predicting the response to stimulus mixtures, even when knowledge of the responses to the individual components of the mixtures were known (Derby and Ache, 1984; Zimmer-Faust et al., 1984; Derby et al., 1985; Gleeson and Ache, 1985; Johnson et al., 1985; Johnson et al., 1989; Borroni et al., 1986; Atema et al., 1989; Carr and Derby, 1986b; Carr and Derby, 1986a). A recent report by Caprio, Dudek and Robinson (1989), however, clearly showed that olfactory receptor responses to binary and trinary mixtures of amino acids were predictable with knowledge of the relative independence of the

receptor site types obtained from electrophysiological cross-adaptation studies (Caprio and Byrd, 1984). Binary mixtures whose components showed little cross-adaptation initiated enhanced responses compared to those whose components were indicated to interact either with a common receptor site or with receptor sites having highly overlapping specificities. This same report (Caprio et al., 1989) indicated that one mechanism for the response enhancement evoked by particular stimulus mixtures is simply the simultaneous activation of independent receptor site types by different components within the mixture. In addition, the report indicated that evidence for mixture suppression was lacking and suggested that some of the previous reports of this phenomenon could be explained by simple competitive binding among stimuli of different potencies that share the same olfactory receptor binding site type (Gleeson and Ache, 1985; Bell et al., 1987). Thus, the olfactory receptor cells of the channel catfish interacted with mixtures of amino acids predictably, and the observed enhancement of the electro-olfactogram (EOG) and integrated neural responses was dependent upon the relative independence of the respective receptor sites for the component stimuli.

Evidence from previous studies (Caprio and Byrd, 1984; Caprio et al., 1987; Caprio et al., 1989; Bruch and Rulli, 1988) indicated that olfactory receptor sites for basic, acidic, and neutral amino acids, respectively, were different from each other. However, it was unclear, for example, whether neutral amino acids with long side-chains (LCN), containing $\geq$ 3 carbons, bound to a single LCN receptor site type or whether the LCN receptor was actually a number of

different receptor site types having overlapping sensitivities for the LCNs. The present study was designed to answer this question for the neutral, acidic and basic amino acids and to determine whether the principles learned from studying the olfactory response to simple binary and trinary mixtures of amino acids (Caprio et al., 1989) would remain intact for the successful prediction of olfactory receptor responses to more complex mixtures.

This report clearly indicates that: (*a*) acidic, basic, LCN and SCN (neutral amino acids with short side-chains, $\leq$ 2 carbons) amino acids, respectively, bind to a group of highly cross-reactive receptor site types, having highly overlapping specificities, (*b*) the receptor site types for basic and acidic amino acids, respectively, are highly independent from those for neutral amino acids, and (*c*) olfactory receptor responses to complex mixtures of amino acids are experimentally predictable.

MATERIALS AND METHODS

Experimental Animals

Twenty-nine channel catfish, *Ictalurus punctatus*, ranging in weight from 12 to 150g were obtained from a local hatchery, were held in floating cages in a nearby university pond and were fed with commercial catfish chow. Animals brought into the laboratory holding facility were held in aerated, charcoal-filtered water in a 250-liter fiberglass aquarium at approximately 25^{o}C. The fish were maintained on a 12:12 light-dark regime, and were used experimentally within two weeks of laboratory holding time (Tucker, 1973).

Animal Immobilization and Anesthesia

The catfish tested were immobilized with an intramuscular injection of Flaxedil (gallamine triethiodide; 0.05 mg/100 g body weight), wrapped in wet tissue paper and secured to a wax block held in a Plexiglass container. The gills were irrigated throughout the experiment with aerated, charcoal-filtered tap water (artesian water), containing 0.005% (initial concentration) MS-222 (ethyl-*m*-aminobenzoate methane sulfonic acid). Supplemental doses of Flaxedil were applied to the fish as required.

Stimulus Compounds and Delivery

Nine neutral L-amino acids with long side-chains (LCN) [methionine (Met), histidine (His), norvaline (nVal), valine (Val), ethionine (Eth), norleucine (nLeu), leucine (Leu), glutamic acid-gamma-methyl ester (GME), phenylalanine (Phe)], five neutral amino acids with short side-chains (SCN), [glycine (Gly), and the L-isomers of alanine (Ala), serine (Ser), glutamine (Gln), threonine (Thr)], two basic L-amino acids [arginine (Arg) and lysine (Lys)] and two acidic L-amino acids [glutamic acid (Glu) and aspartic acid (Asp)] were chosen as components to form various stimulus mixtures (Bruch and Rulli, 1988; Caprio and Byrd, 1984). Stock solutions of individual amino acids (Sigma grade; Sigma Chemical Co., St. Louis, MO.) were prepared weekly in charcoal-filtered tap water (pH approximately 8.5) and stored at $4^{o}C$. The concentration of the amino acids in their respective stock solutions was 10^{-2} M, except Asp, which was 10^{-3} M. Charcoal-filtered tap water was used to dilute stock solutions to the

desired test concentrations. Concentrations of all amino acid solutions used to form mixtures ranged from 10^{-6} to 10^{-3} M. The pH values of all individual amino acid solutions tested remained between 8.0 and 8.5.

Charcoal-filtered tap water continuously bathed the olfactory mucosa at a flow rate of 12 ml/min for EOG recordings and 5 ml/min for integrated neural recordings. Stimulus solutions were introduced into a 0.5 ml Teflon loop of a manual sample injection valve (Omnifit LTD., Atlantic Beach, NY.) and were injected into the water bathing the olfactory organ. Photodensitometry studies indicated that the maximum stimulus concentration delivered to the olfactory mucosa was 75% of the concentration injected (values in the text are the undiluted concentration). The water control was taken from the same charcoal-filtered tap water source as that used to prepare the stimulus solutions. Inter-stimulus intervals were 3 min. Due to the tendency of EOG responses to gradually increase over the course of the experiment, one amino acid (10^{-5} M Met for the LCN tests; 10^{-5} M Ala for the SCN tests) was chosen as the standard stimulus and was regularly applied to trace any changes in responsiveness in the electrophysiological preparation throughout the experiment.

Mixture Definitions and the MDI Index

Two types of mixtures in this report were multimixtures (M) and binary mixtures (B). Since competitive binding among the components of a mixture that have widely different binding affinities could possibly diminish the stimulatory potency of the more potent stimulus(i) (Gleeson and Ache, 1985), the components of both types of

mixtures were adjusted in concentrations to provide approximately equal EOG and neural response magnitudes, respectively. In addition, within each mixture type, there were "within-group" and "across-group" mixtures (Caprio and Byrd, 1984; Caprio et al., 1989). Further, dose-response (D/R) power functions for EOG and integrated neural responses, respectively, to amino acids in the channel catfish (Caprio, 1978; Byrd and Caprio, 1982) were approximately parallel, a criterion necessary for using the mixture discrimination index (MDI) in the present experimental paradigm. Thus, equal dilutions of equipotent stimuli remained equally stimulatory within the range of concentrations (micromolar to millimolar) tested. Calculated for each mixture tested was the MDI, defined as the response to the mixture divided by the average of the responses to the approximately equipotent stimuli that were mixed in equal aliquots to form the corresponding mixture (Hyman and Frank, 1980; Caprio et al., 1989). Theoretically, the MDI equals 1 if the components of the mixture bind to the same olfactory receptor sites and thus would not be distinguished by the system. An MDI significantly < 1 is indicative of mixture suppression, whereas an MDI significantly > 1 indicates that the components bind to different receptor sites and thus may be distinguished by the system.

Multimixtures were formed by mixing equal aliquots of 3-10 equally stimulatory solutions of single amino acids. Thus, the concentration of each component within a multimixture composed of n amino acids was n^{-1} of its original concentration. "Within-group" mixtures of amino acids were mixtures whose components were indicated

from previous electrophysiological cross-adaptation (Caprio and Byrd, 1984) and receptor binding (Bruch and Rulli, 1988) studies to compete for the same receptor site type or highly cross-reactive sites. All "within-group" multimixtures in the present experiments were composed of 3-9 LCNs and 3-5 SCNs, respectively. "Across-group" mixtures of amino acids were mixtures whose components were indicated to interact with relatively independent receptor site types (Caprio and Byrd, 1984; Bruch and Rulli, 1988). "Across-group" multimixtures consisted of a single basic (Arg or Lys), acidic (Glu or Asp) or SCN (Ala or Thr) amino acid with 2-9 LCNs, or a single basic, acidic or LCN (Met or His) amino acid with 2-5 SCNs..pa

Binary mixtures were tested to determine the effects on the MDI of multiple neutral amino acids substituting for a single neutral amino acid and were formed by mixing equal aliquots of two equally stimulatory solutions of amino acids. Solution one was composed of 1-9 neutral amino acids, and solution two comprised a single "across-group" amino acid. Thus, the concentration of each neutral amino acid in a final binary mixture composed of n neutral amino acids was $(2n)^{-1}$ of its original concentration. The concentration of the single "across-group" amino acid in the final mixture was 0.5 of its original concentration. Tested in these experiments were within-group binary mixtures composed of two acidic, basic, LCN, and SCN amino acids, respectively. All across-group binary mixtures in this report consisted of (a) a single basic (Arg or Lys), acidic (Glu or Asp), or SCN (Ala or Thr) solution combined with that of a single LCN or with an all LCN (n=2-9) amino acid mixture, and (2) a single basic, acidic

or LCN (Met or His) solution combined with that of a single SCN or with an all SCN (n=2-5) amino acid mixture.

Recording Techniques and the Response Measure

The olfactory lamellae were exposed by removing the skin, connective tissue, and cartilage dorsal to the nasal capsule. The underwater EOG, a slow negative potential change in response to chemical stimulation, was recorded *in vivo* in twenty-four catfish as described by Silver, Caprio, Blackwell and Tucker (1976) with calomel electrodes via Ringer-agar filled capillary pipettes in the water that continuously bathed the olfactory organ. The EOG response was amplified by a direct-coupled amplifier, displayed on a oscilloscope and recorded on both video and chart recorders. In a subset of experiments, integrated neural activity was recorded *in vivo* in five catfish with metal-filled glass capillary electrodes as described previously (Erickson and Caprio, 1984; Caprio et al., 1989).

The magnitude of both the EOG and the integrated neural responses was measured as the peak height in mm of the phasic displacement from baseline, and all responses to amino acids were adjusted by subtraction of the mean control response. Prior experiments (Caprio, 1980; Evans and Hara, 1985) clearly indicated that differences in the waveform of the underwater EOG were due primarily only to experimental variables affecting the duration of the stimulus flow over the receptors (i.e. both the EOG and the integrated neural activity show sustained tonic activity to the continuous presentation of a stimulus). Thus, the position of the fish in the recording setup along with the surgery to remove the tissue covering

the olfactory organ could affect the clearance rate of the stimulus from the olfactory capsule and thereby change the duration of stimulus contact with the receptors. Although slight changes in the EOG and integrated neural recordings sometimes occurred during a recording session in the same fish, both the phasic EOG and the phasic integrated neural responses were the portions of the signal that were most reproducible.

Data Standardization

Over the course of the experiment (six hours on the average), the EOG response to standard stimuli (either 10^{-5} M Met for the LCN or 10^{-5} M Ala for the SCN tests) gradually, but steadily increased. The mechanism accounting for this phenomenon is unknown; however, it may have been due to the continuous rinsing away of ions from the mucus layer overlying the epithelium of the olfactory organ, thus increasing the resistance which resulted in a greater EOG magnitude. Also due to the slight movements of the electrode or the fish, the magnitude of the integrated neural responses to standard stimuli sometimes changed slightly over time. Thus, in order to minimize the possibility that any response enhancements observed were due to extraneous factors, two standardization procedures were developed to correct for these changes in responses over time. Both procedures employed were based on the same assumption that the change in the response magnitude was parallel in all component amino acids tested at equipotency [i.e. amino acid stimuli have approximately parallel dose-response functions (Caprio, 1978; Silver, 1982)]. The first standardization procedure was used to adjust for slight changes (generally less than $\pm$5% of the initial

standard response) over a short period ($\leq$ 30 min) in the responses to stimuli which were bracketed by responses to two standard stimuli. For example, if there were N test stimuli bracketed by two standard stimuli and the response magnitude was X mm to the first standard stimulus and Y mm to the second, the response magnitude to the first test stimulus was standardized by subtracting (Y-X)/N mm from the measured magnitude; the response magnitude to the second test stimulus was standardized by subtracting 2[(Y-X)/N] mm, and so on up to the Nth test stimulus, in which (Y-X) mm was subtracted from the measured response.

To calculate the MDI values for binary mixtures, the first procedure was sufficient since binary mixtures and their components were applied within two adjacent standard stimuli. However, multimixtures and their components were applied up to six hours apart. During this time period, slight changes (generally less than $\pm$10% of the initial standard response) in the amplitude of the responses to the standard stimuli occurred. Thus, the second standardization procedure was used to adjust the magnitudes among responses to the corresponding multimixtures and their components. The standardization procedure was the same as in the first procedure with the exception that the second procedure corrected for the changes in responses that were not bracketed by two adjacent standard responses. For example, if the response to the standard was X mm prior to testing the component amino acids, and the response to the standard was Y mm when applied close in time to the response to the multimixture, then the standardization procedure involved adding (Y-X) mm to the average

response magnitude for the component amino acids. This latter procedure ensured that the MDI value for the response to the multimixture was not artificially elevated by slow increases in response magnitudes that generally occurred in the preparation over time. All MDI values presented in this study were calculated from the corrected response magnitudes.

Data Analysis

The data were analyzed with a one-way ANOVA using SAS (1986, SAS Institute Inc., Cary, North Carolina). Means were further analyzed using Waller-Duncan K-ratio *t*-test.

RESULTS

EOG: "Within-group" Multimixtures

From one to nine equipotent LCNs (Fig. I.1A) and one to five equipotent SCNs, respectively, were used to form mixtures with sequentially increasing number of components to determine the relationship of EOG magnitude to the number of "within-group" components in the respective mixtures. Of three different orders (i.e., different arrangements) of the LCNs and SCNs tested to form the series of "within-group" LCN (Table I.1) and SCN (Table I.2) mixtures, respectively, mean MDI values for the resulting mixtures consisting of equal number of components were not significantly different. This occurred despite wide differences in total molarity of the resulting amino acid mixtures. Thus, for the following determinations, all MDI values derived from the "within-group" LCN and SCN mixtures, respectively, across the three orders were pooled.

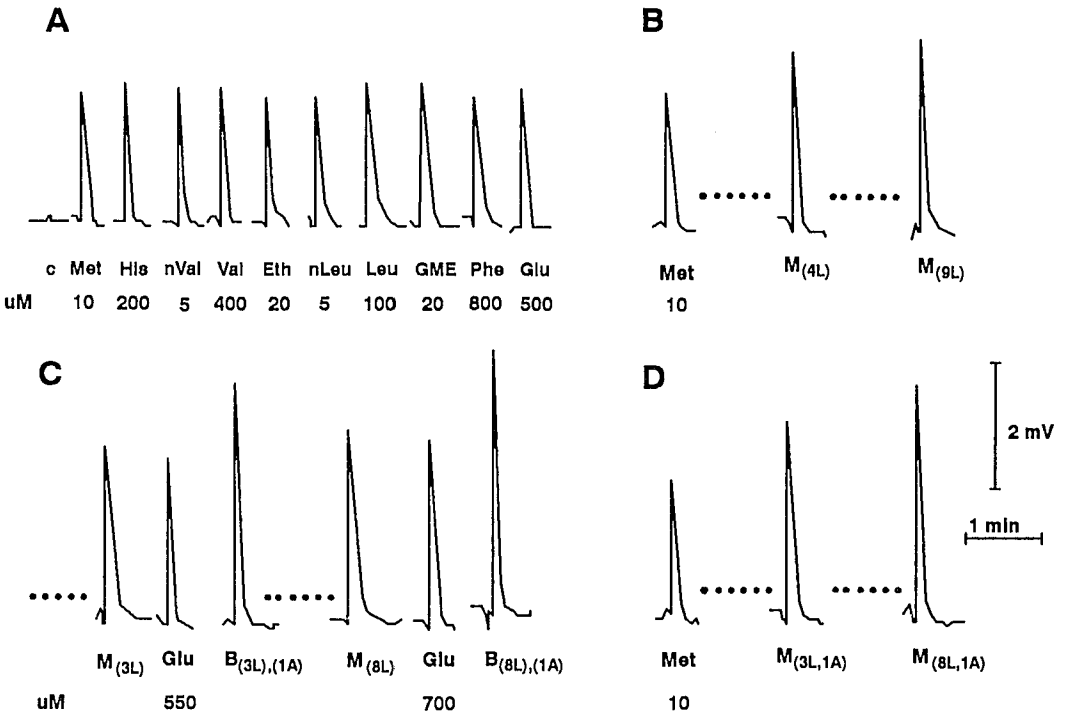


Fig. I.1. Representative EOG responses to single amino acids and to their mixtures. (*A*) EOG responses to control c, L-isomers of nine single LCNs and an acidic amino acid (Glu) that were adjusted in concentration (listed below each stimulus) to provide for approximately equal response magnitudes. (*B*) EOG responses to Met and two "within-group" multimixtures consisting of Met and three additional LCNs from A [i.e. $M_{(4L)}$], and Met and eight additional LCNs from A [i.e. $M_{(9L)}$], respectively. (*C*) Illustrates how four-component [$B_{(3L),(1A)}$] and nine-component [$B_{(8L),(1A)}$] "across-group" binary mixtures were formed. For the four-component mixture, the acidic amino acid, i.e. Glu, was adjusted in concentration to approximately match the response of a three-component, "within-group" multimixture [$M_{(3L)}$]; equal aliquots of the preceding two solutions were then mixed together and the EOG response to the resulting mixture was recorded. The nine-component, "across-group" binary mixture was similarly formed. (*D*) EOG responses to Met and two "across-group" multimixtures. A comparison of B and D illustrates the responses between "within"- (B) and "across"- (D) group four- and nine-component multimixtures composed primarily of LCN amino acids.

Table I.1. *One-way ANOVA Analysis for the EOG Derived MDI Values Obtained from Three Orders of "Within-group" LCN Mixtures*

Stimuli*	No. of LCNs	Orders			p**
		I	II	III	
Met	1	Met	Met	Met	---
$B_{(1L),(1L)}$	2	His	Phe	nVal	.50
$M_{(3L)}$	3	nVal	GME	Eth	.06
$M_{(4L)}$	4	Val	Leu	Leu	.40
$M_{(5L)}$	5	Eth	nLeu	Phe	.08
$M_{(6L)}$	6	nLeu	Eth	His	.68
$M_{(7L)}$	7	Leu	Val	Val	.82
$M_{(8L)}$	8	GME	nVal	nLeu	.75
$M_{(9L)}$	9	Phe	His	GME	.77
Number of Tests		14	5	3	
Number of Fish		11	5	3	

*$B_{(1L),(1L)}$, a binary mixture formed by mixing equal aliquots of two equipotent LCNs. $M_{(nL)}$, multimixtures formed by mixing equal aliquots of 3-9 equipotent LCNs. Compounds used to form a stimulus mixture included the amino acids listed in each order column from the first LCN (i.e. Met) up to the LCN located in the same row as that mixture [e.g. $M_{(4L)}$ in order II was a mixture formed by mixing equal aliquots of equipotent solutions of Met, Phe, GME and Leu].

**ANOVA analysis shows that within each group of n LCNs, the mean MDI values for any of the three orders were not significantly different from those for mixtures consisting of the equal number of components.

Table I.2. *One-way ANOVA Analysis for the EOG Derived MDI Values Obtained from Three Orders of "Within-group" SCN Mixtures*

Stimuli*	No. of SCNs	Orders			P**
		I	II	III	
Ala	1	Ala	Ala	Ala	---
$B_{(1S),(1S)}$	2	Gly	Thr	Ser	.43
$M_{(3S)}$	3	Ser	Gln	Thr	.13
$M_{(4S)}$	4	Gln	Ser	Gly	.21
$M_{(5S)}$	5	Thr	Gly	Gln	.78
Number of Tests		12	5	4	
Number of Fish		9	4	4	

*$B_{(1S),(1S)}$, a binary-mixture formed by mixing equal aliquots of two equipotent SCNs. $M_{(nS)}$, multi-mixtures formed by mixing equal aliquots of 3-5 equipotent SCNs. Compounds used to form a stimulus mixture included the amino acids listed in each order column from the first SCN (i.e. Ala) up to the SCN located in the same row as that mixture [e.g. $M_{(4S)}$ in order II was a mixture formed by mixing equal aliquots of equipotent solutions of Ala, Thr, Gln and Ser].

**ANOVA analysis showed that within each group of n SCNs, the mean MDI values for any of the three orders were not significantly different from those for mixtures consisting of the equal number of components.

Mean MDI values of "within-group" mixtures comprised of different numbers of LCNs were significantly different (Figs. I.1B, I.2A; Table I.3) (ANOVA, $P<0.0001$). Further analysis (Waller-Duncan *t*-test) indicated a significant increase in the mean MDI values from single and binary LCNs up to five component LCN multimixtures (Table I.3). No further significant increase in the mean MDI values occurred for five- to eight-component LCN multimixtures (Table I.3). The MDI value for an LCN multimixture, consisting of nine components [$M_{(9L)}$], the most complex "within-group" multimixture tested (Figs. I.1B, I.2A), however, was significantly larger than those for the binary LCNs, and the three-, four- and five- component LCN multimixtures. The regression equation derived from 92 tests (N) that describes the general increase in MDI magnitude for the two- to five-component (n) "within-group" LCN multimixtures (including the binary LCN mixture) is:

$$MDI = 0.06n + 1.03 \quad (n=2\text{-}5;\ N=92,\ P<0.0001) \qquad (1)$$

A significant increase in the mean MDI values occurred from single and binary SCNs up to four component SCN multimixtures (Fig. I.2B; Table I.4). The mean MDI for five-component SCN multimixtures was not significantly different from that for the four-component SCN mixture. The regression equation derived from 63 tests (N) that describes the general increase in MDI magnitude for the two- to four-component (n) "within-group" SCN multimixtures (including the binary SCN mixture) is:

$$MDI = 0.06n + 0.97 \quad (n=2\text{-}4;\ N=63,\ P<0.001) \qquad (2)$$

The probability values for the regression equations 1 and 2 indicate

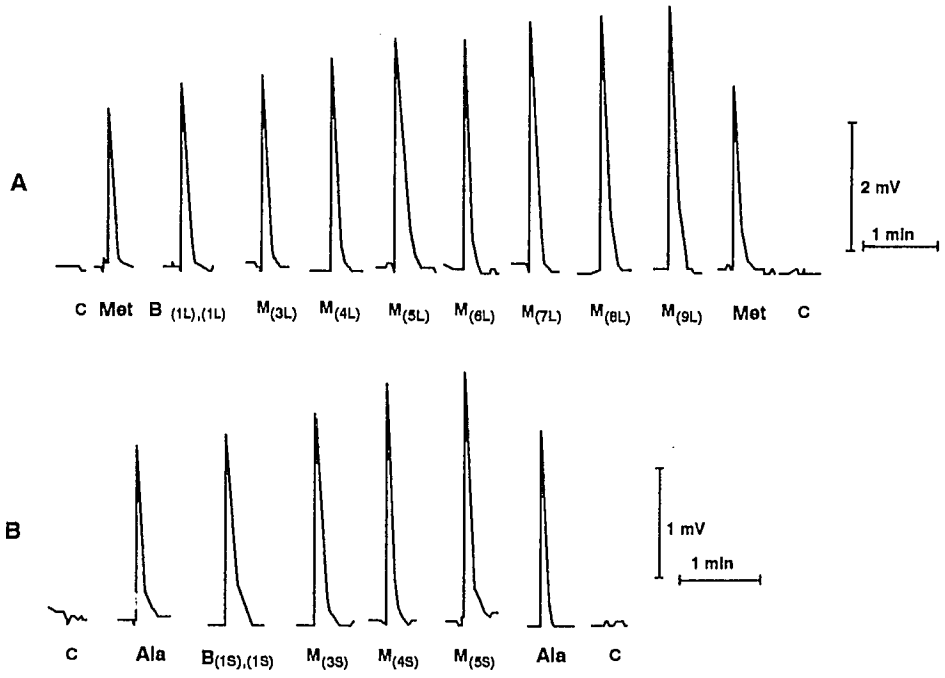


Fig. I.2. Representative EOG responses to "within-group" binary mixtures and multimixtures of neutral amino acids. *(A)* EOG responses to Met, a binary LCN mixture, and sequentially increasing "within-group" LCN multimixtures. *(B)* EOG responses to Ala, a binary SCN mixture, and sequentially increasing "within-group SCN multimixtures. C, indicates the control response. All LCNs and SCNs used to form the respective mixtures were initially adjusted in concentration to result in approximately equal EOG response magnitudes.

Table I.3. *Mean EOG Derived MDI Values for "Within-Group" LCN Mixtures*

Stimuli*	Number of LCNs	MDI (Mean ± SE)	t-test**	
Met	1	1.00 ± 0.00	A	
$B_{(1L),(1L)}$	2	1.14 ± 0.01	B	
$M_{(3L)}$	3	1.22 ± 0.02	C	
$M_{(4L)}$	4	1.27 ± 0.02	D	
$M_{(5L)}$	5	1.32 ± 0.02		E
$M_{(6L)}$	6	1.34 ± 0.02	F	E
$M_{(7L)}$	7	1.36 ± 0.02	F	E
$M_{(8L)}$	8	1.36 ± 0.02	F	E
$M_{(9L)}$	9	1.40 ± 0.02	F	

*Stimuli consisted of a single LCN (i.e. Met), a binary mixture, $[B_{(1L),(1L)}]$, and multimixtures consisting of 3-9 equipotent LCNs, $[M_{(nL)}]$. The MDI value for Met and each of the stimulus mixtures was based on twenty-three tests from a total of thirteen fish.

**One-way ANOVA indicated that there were significant differences ($P<0.0001$) among MDI values for different stimuli. Further *t*-test analysis showed that the mean MDI values with the same letter were not significantly different from each other, but were significantly different ($P<0.05$) from the mean MDI values with different letters.

Table I.4. *Mean EOG Derived MDI Values for "Within-Group" SCN Mixtures*

Stimuli*	Number of SCNs	MDI (Mean ± SE)	*t*-test**
Ala	1	1.00 ± 0.00	**A**
$B_{(1S),(1S)}$	2	1.09 ± 0.01	**B**
$M_{(3S)}$	3	1.15 ± 0.01	**C**
$M_{(4S)}$	4	1.21 ± 0.02	**D**
$M_{(5S)}$	5	1.22 ± 0.02	**D**

*Stimuli consisted of a single SCN (i.e. Ala), a binary mixture, $[B_{(1S),(1S)}]$, and multimixtures consisting of 3-5 equipotent SCNs, $[M_{(nS)}]$. The MDI value for Ala and each of the stimulus mixtures was based on twenty-one tests from a total of seven fish.

**One-way ANOVA indicated that there were significant differences ($P<0.0001$) among MDI values for different stimuli. Further *t*-test analysis showed that the mean MDI values with the same letter were not significantly different from each other, but were significantly different ($P<0.05$) from the mean MDI values with different letters.

that the MDI values reported here are due to the experimental manipulations and that the equations can confidently be used to predict the MDI values for LCN and SCN multimixtures, respectively, for EOG responses in channel catfish.

EOG: "Across-group" Multimixtures

Three sequences of "across-group" mixtures, $M_{(nL,1B)}$, $M_{(nL,1A)}$ and $M_{(nL,1S)}$, formed by mixing a basic (Arg or Lys), acidic (Glu or Asp), or SCN (Ala or Thr) amino acid with a sequentially increasing number of LCN components, were tested (Fig. I.3). For mixtures with equal number of components, MDI values for $M_{(nL,1B)}$ and $M_{(nL,1A)}$, respectively, were significantly greater than those for $M_{(nL,1S)}$. In addition, MDI values for the former two sequences of mixtures were significantly greater than those for the "within-group" LCN mixtures, consisting of the equal total number of amino acids. The sole exception was that the MDI value for $M_{(8L,1B)}$ was not significantly different from those for $M_{(8L,1S)}$ and $M_{(9L)}$. However, the MDI values for the $M_{(nL,1S)}$ and the "within-group" LCN mixtures consisting of the equal number of components were not significantly different from each other. Within each of the three sequences, MDI values generally increased with the increasing number of LCN components in the "across-group" mixtures (Figs. I.1D, I.3). The regression equations derived from 50, 24 and 30 tests (N), respectively, that describe this increase in MDI magnitude for each of the three sequences of two- to six-component (n) "across-group" multimixtures, including the binary mixtures comprising one LCN and one "across-group" component, are:

for $M_{(nL,1B)}$, MDI = 0.03n + 1.28 (n=2-6; N=50, $P<0.001$) (3)

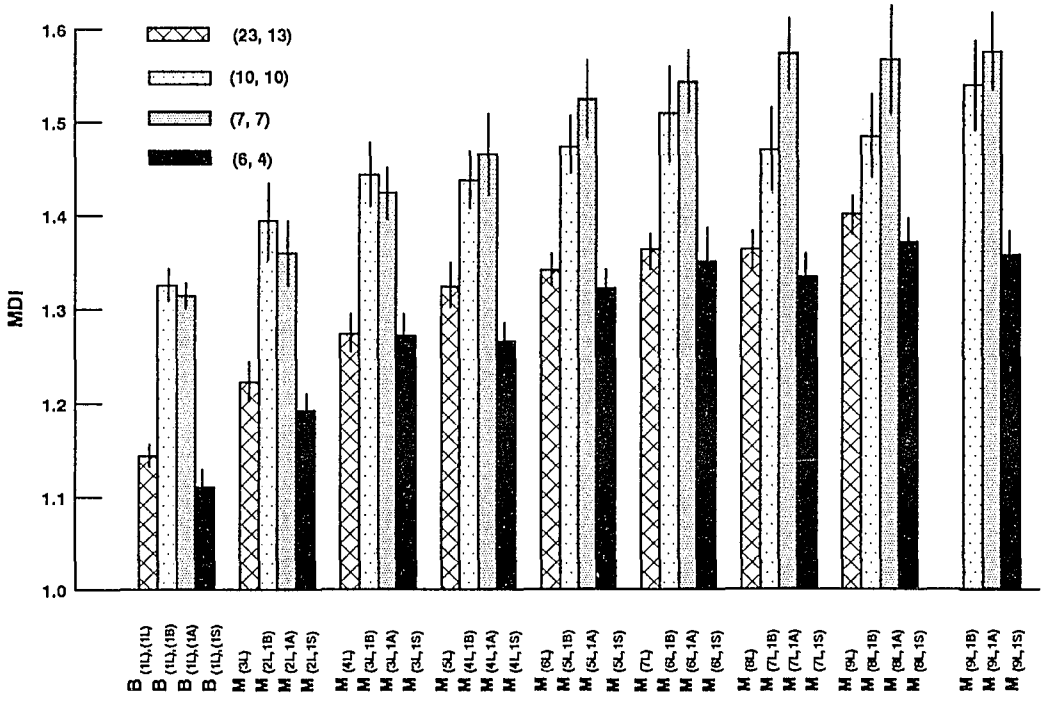


Fig. I.3. Comparison of mean (± SE) MDI values for two-component binary mixtures (B) and three- to ten-component multimixtures (M) composed primarily of LCN amino acids. Numbers in brackets indicate number of tests and fish, respectively.

for $M_{(nL,1A)}$, MDI = 0.05n + 1.21 (n=2-6; N=24, P<0.0001) (4)

for $M_{(nL,1S)}$, MDI = 0.05n + 1.03 (n=2-6; N=30, P<0.0001) (5)

Similarly, MDI values for the "across-group" mixtures, $M_{(nS,1B)}$ and $M_{(nS,1A)}$, were significantly greater than those for the "across-group" mixtures, $M_{(nS,1L)}$, and those for the "within-group" SCN mixtures consisting of the equal number of components (Fig. I.4). However, MDI values for $M_{(nS,1L)}$ were not significantly different from those for the "within-group" SCN mixtures comprising the equal number of components. Within each of the three sequences, MDI values generally increased with an increasing number of SCNs in the "across-group" mixtures (Fig. I.4). The regression equations derived from 40, 20 and 24 tests (N), respectively, that describe this increase in MDI magnitude for each of the three sequences of two- to five-component (n) "across-group" multimixtures, including the binary mixtures comprising one SCN and one "across-group" component, are:

for $M_{(nS,1B)}$, MDI = 0.02n + 1.25 (n=2-5; N=40, P<0.05) (6)

for $M_{(nS,1A)}$, MDI = 0.03n + 1.25 (n=2-5; N=20, P<0.001) (7)

for $M_{(nS,1L)}$, MDI = 0.04n + 1.03 (n=2-5; N=24, P<0.001) (8)

The P values in equations 3-8 indicate that the MDI values for "across-group" multimixtures are highly correlated to the number of amino acids forming the mixtures. Thus, these equations predict the MDI values for the "across-group" multimixtures composed primarily of LCN (equations 3-5) and SCN (equation 6-8) amino acids, respectively.

EOG: "Within-group" Binary Mixtures

For acidic and basic amino acids, respectively, only two-component "within-group" mixtures were tested. MDI means (± SE) for

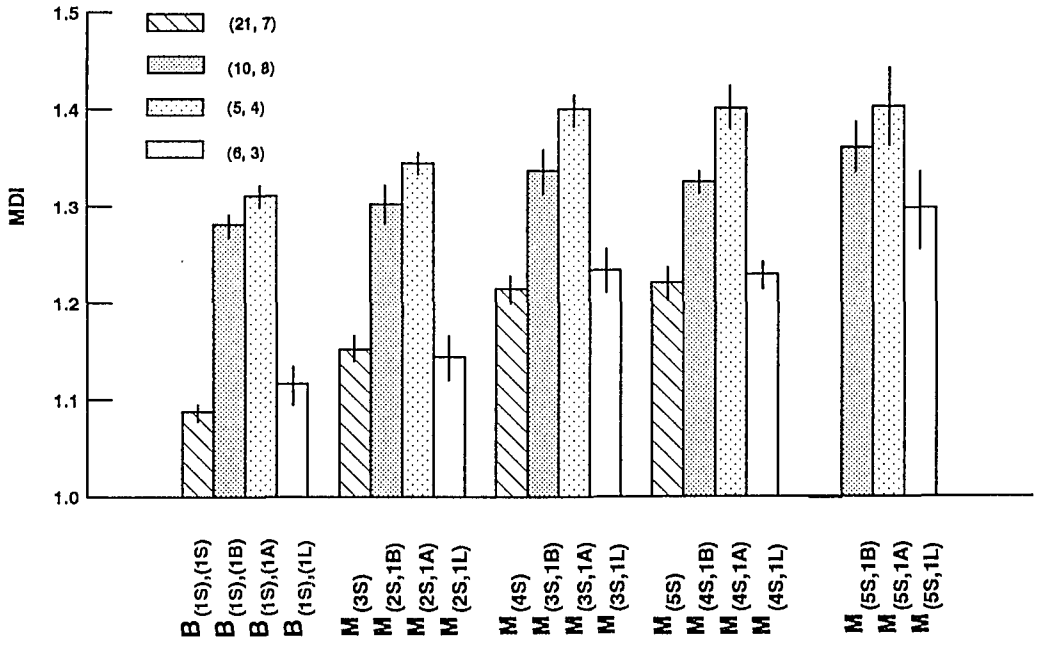


Fig. I.4. Comparison of mean (± SE) MDI values for two-component binary mixtures (B) and three- to six-component multimixtures (M) composed primarily of SCN amino acids. Numbers in brackets indicate number of tests and fish, respectively.

the EOG responses to "within-group" binary mixtures of acidic (L-glutamic and L-aspartic acids) and basic (L-arginine and L-lysine) amino acids, respectively, were 1.10±0.02 (n=8) and 1.16±0.01 (n=8), with both values being significantly greater than one. Similarly, MDI means (± SE) for the "within-group" binary mixtures composed of two LCN and two SCN amino acids, respectively, were 1.14±0.01 (n=23, Table I.3) and 1.09±0.01 (n=21, Table I.4), which were also significantly greater than one.

EOG: "Across-group" Binary Mixtures

Of the three sequences of "across-group" binary mixtures, $B_{(nL),(1B)}$, $B_{(nL),(1A)}$ and $B_{(nL),(1S)}$, mean MDI values for the former two sequences of binary mixtures were significantly greater than those for the latter sequence and ranged between 1.19 and 1.32 (mean±SE, 1.24±0.01) for the $B_{(nL),(1B)}$ sequence, and between 1.26 and 1.31 (mean±SE, 1.29±0.01) for the $B_{(nL),(1A)}$ sequence (Fig. I.5). Mean MDI values for the sequence of "across-group" binary mixtures consisting of a single SCN added to stimuli with increasing numbers of LCN components, i.e. $B_{(nL),(1S)}$, ranged between 1.06 and 1.11 (mean±SE, 1.09±0.01). There were no significant changes ($P>0.05$) among the respective MDI values for "across-group" mixtures formed by the binary mixing of sequentially increasing numbers of LCN components with a single acidic amino acid [$B_{(nL),(1A)}$] or SCN [$B_{(nL),(1S)}$]; however, MDI values for the binary mixing of sequentially increasing numbers of LCN components with a basic amino acid [$B_{(nL),(1B)}$] decreased significantly ($P<0.001$; Fig. I.5). The MDI values for the mixtures, $B_{(2L),(1B)}$ and $B_{(3L),(1B)}$ were not significantly different

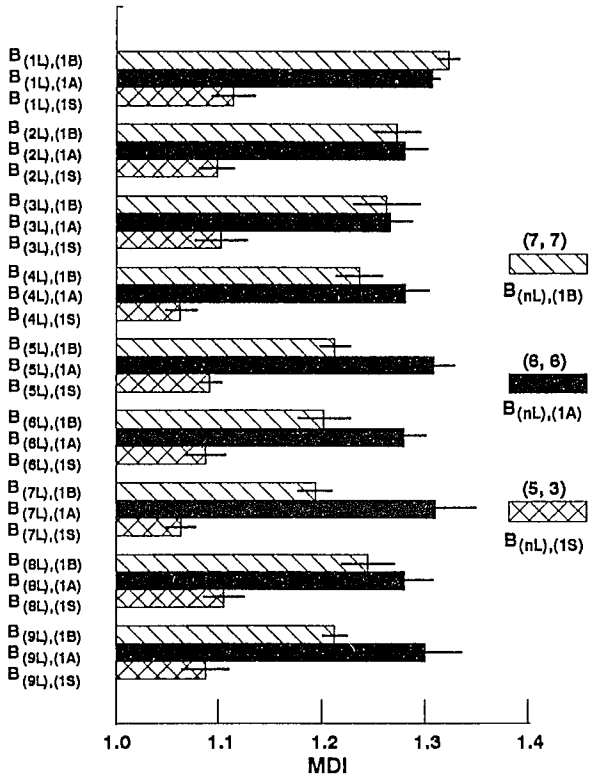


Fig. I.5. Comparison of mean (± SE) MDI values for two- to ten-component "across-group" binary mixtures: $B_{(nL),(1B)}$, $B_{(nL),(1A)}$ and $B_{(nL),(1S)}$. Numbers in brackets indicate number of tests and fish, respectively.

from $B_{(1L),(1B)}$, but the MDI values for mixtures, from $B_{(4L),(1B)}$ to $B_{(9L),(1B)}$, were all significantly smaller than $B_{(1L),(1B)}$ (Fig. I.5).

Similarly for binary mixtures involving SCN stimuli, MDI values for $B_{(nS),(1B)}$ and $B_{(nS),(1A)}$ were significantly greater than those for $B_{(nS),(1L)}$ and ranged between 1.27 and 1.29 (mean±SE, 1.28±0.00) for $B_{(nS),(1B)}$, and between 1.19 and 1.31 (mean±SE, 1.25±0.02) for $B_{(nS),(1A)}$ (Fig. I.6). MDI values for the sequence of "across-group" mixtures consisting of a single LCN added to stimuli with increasing numbers of SCN components, $B_{(nS),(1L)}$, ranged between 1.08 and 1.12 (mean±SE, 1.11±0.01). There were no significant changes ($P>0.05$) among the respective MDI values for "across-group" mixtures formed by the binary mixing of sequentially increasing numbers of SCN components with a single basic amino acid [$B_{(nS),(1B)}$] or LCN [$B_{(nS),(1L)}$]; however, MDI values for the binary mixing of the SCN sequences with an acidic amino acid [$B_{(nS),(1A)}$] decreased significantly ($P<0.01$). MDI values were not significantly different between $B_{(1S),(1A)}$ and $B_{(2S),(1A)}$, but the MDI values for $B_{(3S),(1A)}$, $B_{(4S),(1A)}$ and $B_{(5S),(1A)}$ were all significantly smaller than that for $B_{(1S),(1A)}$ (Fig. I.6).

Neural Activity

Eleven different mixtures, two "within-group" and three "across-group" multimixtures, and one "within-group" and five "across-group" binary mixtures were selected to confirm that the enhanced EOG responses to mixtures were of neural origin and were mirrored in the action potential activities of olfactory receptor neurons. As previously indicated for the EOG recordings, "across-group"

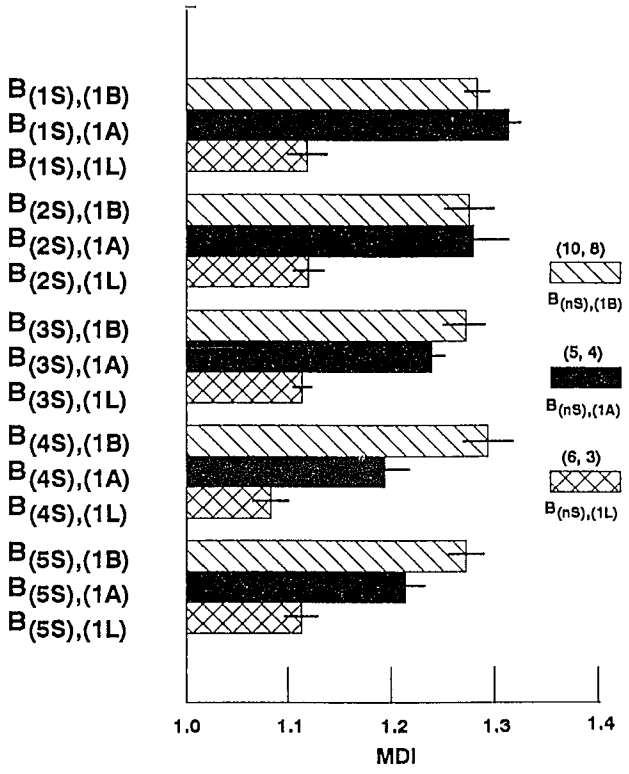


Fig. I.6. Comparison of mean (± SE) MDI values for two- to six-component "across-group" binary mixtures: $B_{(nS),(1B)}$, $B_{(nS),(1A)}$ and $B_{(nS),(1L)}$. Numbers in brackets indicate number of tests and fish, respectively.

multimixtures and binary mixtures, composed of a basic or acidic amino acid and neutral amino acids, resulted in significantly larger MDIs than occurred for "within-group" multimixtures and binary mixtures, respectively (Table I.5). The neurally-derived MDI value for a "within-group" binary mixture, i.e. $B_{(1L),(1L)}$, was not significantly different than that for the "across-group" binary mixture, $B_{(1L),(1S)}$, as was also predicted by the EOG recordings. Further, the finding for the EOG derived data that there was no significant change in the MDI values with increasing numbers of LCN compounds for the binary mixtures, $B_{(nL),(1A)}$, also occurred for the neurally-derived MDIs [compare $B_{(1L),(1A)}$ with $B_{(6L),(1A)}$]. For the binary mixtures involving a basic amino acid, i.e. $B_{(nL),(1B)}$, the neurally-derived MDI values were also consistent with the EOG derived data [compare $B_{(4L),(1B)}$ with $B_{(6L),(1B)}$]. The neurally-derived MDI values for three "within-group" mixtures, i.e. $B_{(1L),(1L)}$, $M_{(4L)}$ and $M_{(6L)}$, increased with increasing numbers of LCN compounds in the mixtures (Table I.5). However, significant changes in the neurally-derived MDI values occurred only between those for $B_{(1L),(1L)}$ and $M_{(4L)}$, and $B_{(1L),(1L)}$ and $M_{(6L)}$, as in the EOG recordings, but not between those for $M_{(4L)}$ and $M_{(6L)}$ (Table I.5).

DISCUSSION

Receptor Site Types for Amino Acids

The question as to how many different receptor site types for amino acid stimuli are present within the membranes of olfactory receptor neurons in fish is still an unsolved question, and may possibly be species dependent; however, electrophysiological (Caprio

Table I.5. *Comparison of EOG and Neurally Derived MDI Values*

Stimuli	EOG			Neural		
	n_1*	n_2*	MDI (Mean±SE)	n_1*	n_2*	MDI (Mean±SE)
***A.* Multimixtures**						
$M_{(4L)}$	23	13	1.27 ± 0.02	4	2	1.36 ± 0.03
$M_{(6L)}$	23	13	1.34 ± 0.02	13	3	1.36 ± 0.03
$M_{(3L,1B)}$	10	10	1.44 ± 0.04	4	2	1.57 ± 0.07
$M_{(5L,1B)}$	10	10	1.47 ± 0.03	4	2	1.63 ± 0.06
$M_{(5L,1A)}$	7	7	1.53 ± 0.11	5	3	1.65 ± 0.09
***B.* Binary mixtures**						
$B_{(1L),(1L)}$	23	13	1.14 ± 0.01	5	3	1.20 ± 0.07
$B_{(1L),(1S)}$	6	4	1.11 ± 0.02	4	2	1.13 ± 0.07
$B_{(1L),(1A)}$	7	7	1.32 ± 0.01	3	2	1.61 ± 0.07
$B_{(4L),(1B)}$	7	7	1.23 ± 0.02	2	1	1.55 ± 0.05
$B_{(6L),(1B)}$	7	7	1.20 ± 0.03	6	3	1.56 ± 0.07
$B_{(6L),(1A)}$	6	6	1.28 ± 0.02	6	3	1.52 ± 0.06

*n_1, Number of tests; n_2, Number of fish.

and Byrd, 1984; Ohno et al., 1984; Sveinsson and Hara, 1990a; Sveinsson and Hara, 1990b) and biochemical (Cagan and Zeiger, 1978; Cancalon, 1978; Novoselov et al., 1980; Brown and Hara, 1981; Rhein and Cagan, 1983; Rehnberg and Schreck, 1986; Kalinoski et al., 1987; Bruch and Rulli, 1988) studies have provided critical evidence to indicate that multiple olfactory receptor sites for amino acids exist. Based on electrophysiological cross-adaptation experiments in the channel catfish, Caprio and Byrd (1984) suggested the relative independence of receptor sites for the acidic, basic and neutral amino acids. The independence of the acidic and basic sites from the neutral sites was subsequently confirmed biochemically by receptor binding experiments (Bruch and Rulli, 1988). Bruch and Rulli (1988) also confirmed the existence of previously identified (Caprio and Byrd, 1984) receptor sites (SCN) that selectively recognize neutral amino acids with short side-chains. As a group, the LCN ligands (neutral amino acids with long side-chains) were significantly different from SCN ligands in their ability to compete with L-[^{3}H]alanine for specific binding sites in isolated cilia preparations from the olfactory epithelium of the channel catfish. However, the present findings of increasing MDI values for "within-group" LCN and SCN mixtures, respectively (tables I.3 and I.4) suggest that there are multiple sites within both the LCN and SCN categories. Theoretically, if all the components of a mixture are equipotent and bind to the same site, the MDI should remain 1.0. This did not occur. The concept that both LCN and SCN amino acids bind to receptor sites with overlapping specificities is also consistent with the previous

cross-adaptation results that showed EOG responses to some amino acids thought to bind to the same receptor site type were differentially affected by the same adapting stimuli (Caprio and Byrd, 1984).

Since the MDIs for "within-group" mixtures indicate multiple cross-reactive LCN and SCN receptor site types, the question still remains as to the total number of olfactory receptor site types in the channel catfish for the neutral amino acids. Since the increase in the MDI values reached an approximate asymptote at approximately 5 LCN and 4 SCN components, respectively, at least 4-5 types of binding sites exist for these compounds. It is still possible that a receptor site type exists with the highest affinity for each of the neutral amino acids; however, the high degree of cross-reactivity for other neutral amino acids across these site types might have masked the appearance of additional types in both the biochemical and electrophysiological competition and the mixture experiments. The present study also indicated that MDI values for binary mixtures of acidic and basic amino acids, respectively, ranged between 1.1-1.2, thus suggesting more than one common receptor site for the two commonly occurring acidic and basic amino acids, respectively.

Although in the present report, MDI values for binary mixtures of equipotent "within-group" amino acids were somewhat similar to the earlier report (Caprio et al., 1989), MDI values for binary mixtures in the present experiments were slightly larger and significantly greater than 1.0. This finding was surprising in that the earlier experiments with binary and trinary "within-group" mixtures, MDI

values were not significantly different than 1.0. This seeming conflict in the two studies probably arose due to the different "within-group" binary mixtures tested in the two experiments. For example, L-methionine and L-glutamic acid-gamma-methyl ester, LCN compounds that reciprocally cross-adapted each other (Caprio and Byrd, 1984) and in binary mixtures produced the smallest MDI value of all amino acids examined previously (Caprio et al., 1989), were not tested in the present experiments. Nevertheless, in both experiments, EOG and neurally derived MDI values for "across-group" binary mixtures were similar and significantly greater than those for the "within-group" binary mixtures.

Olfactory Receptor Responses to Complex Mixtures

The present results are in agreement with the earlier study of olfactory receptor responses to binary and trinary mixtures of amino acids (Caprio et al., 1989), in that evidence for mixture suppression was lacking. A number of contemporary reports of olfactory mixture suppression occurring in response to amino acid mixtures in decapod crustaceans (Derby and Ache, 1984; Johnson et al., 1985; Carr and Derby, 1986b; Carr and Derby, 1986a) remains in sharp contrast to the present and past results for the olfactory receptors of the channel catfish. Either the olfactory mechanisms for olfactory mixture detection and receptor processing of amino acid information are different between these species (and possibly between aquatic invertebrates and vertebrates) or differences in the experimental paradigms and/or theoretical bases for defining mixture interactions in the different laboratories involved are the basis for the profound

differences reported. It is our present contention that a number of previous reports of mixture suppression in aquatic organisms were confounded by (1) the experimental conditions of testing stimuli of varying concentrations and potencies without knowledge of the number of relatively independent receptor sites involved, and (2) the criterion that less than an "expected" additivity of the responses to the individual components of the mixture signifies the occurrence of mixture suppression. As pointed out previously (Caprio et al., 1989), this criterion is valid only if the D/R functions to the stimuli are linear. Since olfactory D/R functions for amino acids in both electrophysiological and behavioral assays in teleosts and decapod crustaceans are nonlinear, the additivity criterion is invalid, and has possibly caused an overestimation of the occurrence of mixture suppression. As for possibility 1, competitive binding among stimuli having differing receptor affinities could likely have resulted in the mixture suppression reported (Gleeson and Ache, 1985; Bell et al., 1987). Competitive binding between a strong and weak agonist for an olfactory receptor site, as reported for taurine and glycine in antennular chemoreceptors in *Panulirus argus* (Gleeson and Ache, 1985), would diminish the stimulatory effects of the stronger agonist and be one mechanism for mixture suppression. Since all components used to form mixtures in the experiments in the channel catfish were adjusted for equal potency, this mechanism for mixture suppression was experimentally eliminated.

The major finding by Caprio et al. (1989) of enhanced olfactory receptor activity to binary and trinary mixtures of amino acids whose

components bound to relatively independent receptor sites (Caprio and Byrd, 1984), was confirmed here for more complex mixtures. An enhanced olfactory receptor response is defined in the present context as a response to a mixture that is larger than that produced by a "within-group" mixture consisting of the same total number of components. Whenever a basic or acidic amino acid was mixed either in a 50:50 proportion with an equipotent mixture of up to nine neutral amino acids (i.e. in "binary" mixtures) or in a declining proportion with increasing number of equipotent neutral amino acids (i.e. in multimixtures), the response to the resulting mixture was significantly larger than the magnitude of the component solutions used to form the mixture. As previously suggested (Caprio et al., 1989), this response enhancement that occurred by mixing stimuli that bind to relatively independent receptor sites may be one mechanism of synergism. Thus, much of the difficulty reported in earlier attempts to predict responses of stimulus mixtures from experimentally-derived responses to the individual components may be attributed to insufficient information as to the relative independence of the respective receptor sites for the component stimuli.

Theoretically, the MDI of even a 10-component "binary" "across-group" mixture, as formulated in the present experiments (i.e. the mixing of equivolumes of two equipotent solutions, one composed of a single basic or acidic amino acid and the second solution composed of 2-9 neutral amino acids), should be similar (not significantly different) to that of a two-component, binary "across-group" mixture. Although this occurred for all "binary" "across-group" mixtures in

which a solution of LCNs was mixed with an acidic amino acid, it did not occur for an LCN solution mixed with a basic amino acid. Conversely, MDIs were similar for "binary" "across-group" mixtures in which a solution of SCNs was mixed with a basic, but not with an acidic amino acid. In both the exceptions, the MDI decreased with an increasing number of neutral amino acid components. Although the exact cause(s) for the reduction in the MDI is (are) presently unknown, the results of cross-adaptation experiments (Caprio and Byrd, 1984) and the results presented here provide for an interesting speculation. In the previous study, a principal components analysis related each test amino acid to all others on the basis of similarities of the EOG responses to the test stimuli across eight different amino acid adapting regimes. This type of analysis placed the neutral amino acids with short side-chains (SCN) close to the acidic amino acids, and the neutral amino acids with long side-chains (LCN) close to the basic amino acids. These results of the cross-adaptation experiments indicated that although the SCN and acidic amino acids bound to relatively independent receptor sites, there were more similarities in the effects of the cross-adapting regimes on these test stimuli than observed between SCN and basic amino acids; conversely, although the cross-adaptation results indicated that LCN and basic amino acids bound to relatively independent sites, there were more similarities in the effects of the cross-adapting regimes on these test stimuli than observed between LCN and acidic amino acids. One possible explanation for these findings is that receptors for (1) SCN and acidic amino acids, and (2) LCN and basic amino acids,

respectively, have a greater probability of being located on the same receptor neurons than being segregated on different receptor neurons. It is also reasonable to assume that different receptor site types present on the same receptor cell when activated by a mixture cannot generate the same total amount of receptor potential as do independent receptor sites located on different receptor cells. Thus, the reduction in the MDIs for the two types of "across-group" binary mixtures in the present results was possibly due to the limitation in output of the individual receptor cells containing relatively independent receptor sites for the respective stimuli.

An enhanced olfactory receptor response was also clearly evident in "across-group" multimixtures, where, with the increasing number of "within-group" components (neutral amino acids), the percent content of the basic or acidic amino acid (the single "across-group" component) declined. This diluting out of the "across-group" component would tend to compromise somewhat the response enhancement that results from activating a relatively independent amino acid site type, while the increase in the number of neutral amino acid components in multimixtures of increasing complexity would tend to increase the MDI (Tables I.3 and I.4). However, the MDI values for the "across-group" multimixtures were still significantly greater than those for the "within-group" multimixtures consisting of the equal total number of amino acids (Figs. I.3 and I.4). The sole exception, the nine-component "across-group" multimixture, $M_{(8L,1B)}$, not resulting in a significantly larger MDI than that for the

"within-group" multimixture, $M_{(9L)}$, (Fig. I.3), is currently unexplained, but may be due to the saturation of the multiple receptor sites.

With an increasing number of neutral amino acid components, the MDI values for the "across-group" binary mixtures remained approximately constant or decreased slightly (Figs. I.5, I.6), while the MDI values for the "across-group" multimixtures increased (Figs. I.3, I.4). However, due to differences in the construction of the respective mixtures, the actual EOG response magnitudes to "across-group" mixtures, consisting of neutral and basic or neutral and acidic amino acids, were greater to the binary mixtures than to the multimixtures. This, however, should be apparent when considering that in the 10-component "across-group" binary mixtures, the single basic or acidic amino acid was 50% of the solution, whereas it was only 10% of the solution in the multimixture.

Predicting Olfactory Receptor Responses to Complex Mixtures

Caprio et al. (1989) used two models, the "stimulus addition" (Cameron, 1947; Bartoshuk and Cleveland, 1977) model, also referred to as the "input summation" (Cain, 1975) or "stimulus summation" (Carr and Derby, 1986b; Carr and Derby, 1986a) model, and the "response summation" (Carr and Derby, 1986a) model, also referred to as the "sum of perceived intensity" (Bartoshuk, 1977) or "output summation" (Cain, 1975) model to predict olfactory receptor responses of channel catfish to stimulus mixtures. In the "stimulus addition" model, the components in a mixture, which bind to the same receptor site and are

therefore indistinguishable by the system, are expressed as "equivalent" concentrations of one of the components, the reference compound. These values for each of the components are then summed and the mixture response is predicted by the D/R function of the reference compound at the higher resulting concentration. For the "response summation" model, the components in a mixture activate independent receptor site types and initiate a mixture response that is the sum of the responses to each of the components applied individually. Based on the results of binary and trinary mixtures of amino acids (Caprio et al., 1989), the "stimulus addition" model appeared adequate to predict the responses to "within-group" mixtures, whereas the "response summation" model was a better choice for predicting "across-group" mixtures. However, since the present results indicate that a number of different and highly overlapping receptor site types, rather than a single receptor type, exist for neutral amino acids (indicated by the increasing MDI for "within-group" mixtures; Tables I.3 and I.4; Figs. I.2-I.4), the "stimulus addition" model underestimates the responses to more complex mixtures of neutral amino acids. An accurate prediction of the response to a mixture consisting of equipotent, within-group neutral amino acids, however, can be formulated by using the experimentally determined regression equations 1 and 2. Also, equations 5 and 8, which predict MDI values for "across-group" multimixtures composed of LCN and SCN amino acids, are similar to equations 1 and 2 which is further evidence that the receptor sites for the LCN and SCN amino acids have highly overlapping specificities.

The results of the "across-group" mixtures in the present and previous report (Caprio et al., 1989) indicate that although the responses to both the "across-group" binary mixtures and multimixtures consisting of neutral amino acids mixed with basic or acidic amino acids are significantly larger than those predicted by the "stimulus addition" model, the "response summation" model overestimates the mixture responses. A probable reason for the responses to these "across-group" mixtures not attaining the predicted values is that the receptors for the basic and acidic amino acids may not be as segregated on different receptor neurons as those for the neutral amino acid components of the mixture. For the multimixtures, a more accurate estimate for "across-group" multimixtures composed of neutral amino acids and a basic or acidic amino acid can be obtained by using the experimentally determined regression equations 3, 4, 6 and 7 for the respective mixture types.

Implications for Natural Mixtures

With certainty, mixtures of amino acids found in nature would be more complex than those tested here. Rarely would a naturally occurring amino acid mixture be found in which the components would be in the appropriate concentrations that would be equipotent to the chemical receptors. Further, what might be equipotent concentrations to one chemosensory system, like olfaction, could be of quite different potencies to another system, like taste, since the relative effectiveness for amino acids differ between the two chemosensory systems of teleosts (Caprio, 1977; Caprio, 1978; Goh and Tamura,

1980). However, what appears essential in this and previous work on amino acid mixtures in teleosts (Caprio et al., 1987; Caprio et al., 1989), is that to be able to successfully predict the responses to any mixture, knowledge of the specificity of the respective binding sites for the components of the mixture is required. Among the components of a mixture, it is necessary to determine which amino acids bind to the same site, to highly overlapping sites, and to relatively independent sites in order to determine the appropriate amplification factor (i.e. the degree of enhancement if it occurs due to "across-group" components in the mixture). It also may be important to know how the independent sites are distributed, whether they are co-localized on receptor cells or are found on different cells. Possibly the amplification factor for two or more independent receptor sites located on the same olfactory receptor neurons would be less than if they were on different receptor cells. Currently, this information is lacking. However, the present and previous (Caprio et al., 1989) reports provide the essential base of information for the further testing of mixtures on single olfactory neurons by patch clamp or other intracellular methods.

Although not yet found in teleosts, certain amino acids applied to antennular chemoreceptors in the southern spiny lobster, *Panulirus argus*, hyperpolarize the membrane and lead to a suppression of activity (Michel and Ache, 1990). Also, other mechanisms of "synergism" other than the activation of relatively independent receptor site types may be found. Since under most conditions in nature chemoreceptors only detect stimulus mixtures, knowledge of the

mechanisms of mixture detection are critical for beginning to understand the process(es) of olfaction.

EOG and Neural Responses

Because of the relatively long-term stability of the preparation, the majority of the data presented were obtained from EOG recordings, although neural spike activity of relatively small populations of olfactory receptors to selected mixtures were also obtained. Even though EOG responses are composed of generator potentials from receptor neurons and non-neural events, such as secretory potentials (Getchell, 1974b), EOG responses to amino acids in teleosts have previously been shown to be reliable indicators of olfactory neural activity to both individual amino acids (Caprio, 1978; Silver, 1982) and to their binary mixtures (Caprio et al., 1989). As in the previous studies in fish, only the phasic displacement of the EOG from baseline was used as a reproducible and thus reliable measure of the magnitude of activation of the olfactory receptor neurons. The varying shape of the EOG and thus the area under the EOG waveform is a reflection of the tonic activity of the receptors, which is primarily only an indicator of the duration of stimulus activation of the receptors and rate of stimulus clearance from the organ which can vary across preparations.

The results (Table I.5) indicated that although the MDI values derived from EOG and neural recordings were correlated, the response enhancement observed from the two different recording methods for many of the "across-group" mixtures was not linearly related. Since (1) the neurally-derived MDI values are greater for "across-group"

mixtures than the EOG derived values, and (2) the slopes of the dose-response (D/R) functions for amino acids based on neural recordings are significantly less than those for the EOG recordings (Caprio, 1978; Byrd and Caprio, 1982), the percent EOG increase is a highly conservative indicator of the magnitude of the enhancement of action potential activity initiated by the olfactory receptor neurons. To better appreciate the difference in response enhancement attained in the EOG and integrated neural recordings, the response enhancement can be equated to the amplification factor necessary to obtain the same MDI value by increasing only the concentration of one of the equipotent components in an "across-group" mixture. For example, for the "across-group" binary mixture, $B_{(6L),(1B)}$, the EOG derived MDI was about 1.2, whereas the MDI derived neurally was about 1.6 (Table I.5). Based on the MDI definition, an MDI of 1.2 indicates that the response to the mixture was 20% greater than the response to any of the equipotent components. Thus, in order to reach a 20% response enhancement level along the D/R curve, the concentration of any of the components would have to be elevated by a factor of 2.3, calculated from the D/R function $R = k(10)^{\log C/\text{gamma}}$ [EOG: gamma=4.54 (Byrd and Caprio, 1982)]. At the same time, this EOG increase is transduced into a neural MDI of 1.6, which indicates the response to the mixture was 60% greater than the response to any of the equipotent components. In order to obtain a 60% increase in neural response, the stimulus concentration would have to be elevated by at least 50 times [neural: gamma=8.33 (Caprio, 1978)]. It is remarkable that this enhancement in olfactory receptor neural activity in the present experiments resulted

from stimulating the system with an appropriate amino acid mixture and not by elevating stimulus concentrations.

Acknowledgements. Reproduced from The Journal of General Physiology, 1991, 98:699-721 by copyright permission of the Rockefeller University Press.

CHAPTER II

RESPONSES OF SINGLE OLFACTORY RECEPTOR NEURONS TO INDIVIDUAL STIMULI AND TO BINARY MIXTURES

INTRODUCTION

Studies of electro-olfactogram (EOG) and integrated multiunit responses of olfactory receptors in different species of fishes clearly established that amino acids are highly potent chemical stimuli (for reviews see Caprio, 1984; 1988a). Olfactory thresholds for amino acids in fishes were reported to be between 10^{-6} M and 10^{-9} M (Byrd and Caprio, 1982; Caprio, 1978; Caprio, 1980; Silver, 1979; DØving and Holmberg, 1974; Sutterlin and Sutterlin, 1971; Suzuki and Tucker, 1971). Although some differences were observed (Zippel et al., 1993; Li and Sorensen, 1992; Goh and Tamura, 1978), the averaged specificity of the population of olfactory receptors across different species of fishes was highly similar, with the more hydrophobic neutral amino acids generally being most potent.

Electrophysiological (Caprio and Byrd, 1984; Caprio et al., 1989; Ohno et al., 1984; Sveinsson and Hara, 1990b; Sveinsson and Hara, 1990a; Kang and Caprio, 1991) and biochemical (Cagan and Zeiger, 1978; Rehnberg and Schreck, 1986; Kalinoski et al., 1987; Bruch and Rulli, 1988) evidence indicated the existence of multiple olfactory receptor sites for amino acids. Since all electrophysiological studies of the amino acid specificities of olfactory receptor neurons (ORNs) in teleosts were performed *in vivo* using multiunit recording techniques, the distribution of these amino acid receptor sites across the individual ORNs was unknown. With the advent of the whole-cell patch-clamp technique (Marty and Neher, 1983), voltage-activated currents from isolated ORNs of coho salmon (Nevitt and Moody, 1992) and channel catfish (Miyamoto et al., 1992) were characterized, but

there were few indications of the chemical specificities of the individual ORNs.

Through the technique of calcium imaging in living cells, Restrepo and colleagues (Restrepo and Boyle, 1991; Restrepo and Teeter, 1990; Restrepo et al., 1990) were first to provide the initial hint that individual ORNs in the channel catfish responded (i.e. with a rapid transient intracellular calcium increase) heterogeneously to amino acids and that a single ORN could respond to four amino acids that were previously indicated to bind to independent receptor sites (Bruch and Rulli, 1988; Kang and Caprio, 1991; Caprio et al., 1989; Caprio and Byrd, 1984). Recently, whole-cell patch-clamp recordings from isolated ORNs from the channel catfish (Ivanova and Caprio, 1993) confirmed the calcium imaging evidence by showing that single ORNs could respond directly to all four (three of which were identical to those tested in the calcium imaging experiments) of the amino acids tested confirming that multiple receptor sites for different stimuli occurred on individual ORNs. These results in the channel catfish are consistent with previous extracellular electrophysiological recordings in different species of tetrapods indicating that single ORNs are not highly specific, but rather have a relatively broad spectrum to different stimuli (Gesteland et al., 1963; Shibuya and Shibuya, 1963; Gesteland et al., 1965; O'Connell and Mozell, 1969; Shibuya and Tucker, 1967; Getchell, 1973; Getchell, 1974a; Duchamp et al., 1974; Holley et al., 1974; Holley and DØving, 1977; Blank and Mozell, 1976; Revial et al., 1978a; Revial et al., 1978b; Revial et al., 1982;

Revial et al., 1983; Sicard and Holley, 1984; Sicard, 1985; Mathews, 1972a).

Due to the relatively low success rate of obtaining channel catfish ORNs that responded to amino acids in either the calcium-imaging [11 of 219 ORNs, (Restrepo and Boyle, 1991)] or the whole-cell patch recordings [13 of 64 ORNs, (Miyamoto et al., 1992); 14 of 90 ORNs, (Ivanova and Caprio, 1993)], the present study was initiated to determine the feasibility of studying the amino acid specificity of single ORNs using classical extracellular recording techniques. Apparently, because of the high density and small size of the ORNs located within the olfactory lamellae along with the general instability of a lamellar organization, only one *in vivo* study of single ORNs in fishes had been reported (Suzuki, 1978). In addition, the development of an *in vivo* ORN recording preparation would allow for a comparison of the response properties of intact ORNs with those of isolated cells, which because of either the isolation procedures or the isolation itself may not actually reflect the response properties of the intact preparation.

The present *in vivo* study was designed to investigate for the first time in any teleost: (1) the response specificity of single ORNs to odorants, (2) the relative occurrence of excitatory and suppressive responses, and (3) the responses of single ORNs to binary mixtures of amino acids. The results indicated that: (a) the majority of the single ORNs responded to more than one of the tested stimuli that were indicated to bind to different receptor sites, (b) single ORNs

responded to the test stimuli with suppressive responses more frequently than with excitatory responses, and (c) no mixture interactions that changed response types from those observed to the individual components were encountered, indicating that the response types were predictable for binary mixtures whose components elicited the same response types.

METHODS AND MATERIALS

Animal Maintenance, Immobilization and Anesthesia

Twenty-two channel catfish, *Ictalurus punctatus*, ranging from 24g to 74g, were held in floating cages in university ponds and were fed commercial catfish chow. Catfish brought into the laboratory's holding facility were maintained at approximately 25°C in aerated, charcoal-filtered artesian water in 250-liter aquaria on a 12:12 light-dark regime, and were used experimentally within two weeks of laboratory holding time (Tucker, 1973).

The catfish tested were immobilized with an intramuscular injection of Flaxedil (gallamine triethiodide; 0.05 mg/100 g body weight), wrapped in wet tissue paper and secured to a wax block held in a Plexiglass container. Supplemental doses of Flaxedil were applied as required. Perfusion of aerated, charcoal-filtered artesian tap water, containing 0.005% (initial concentration) MS-222 (ethyl-*m*-aminobenzoate methane sulfonic acid), over the gills at approximately 500 ml/min throughout the experiment was accomplished by means of a forked teflon tubing which was placed beneath the operculi using a posterior approach.

Stimulus Compounds and Delivery

Stimuli tested were chosen from three groups of chemical compounds (i.e., amino acids, nucleotides and bile salts) which were previously indicated as being important olfactory stimuli in fish (Caprio, 1988; Caprio, 1990; Michel et al., 1988; DØving et al., 1980; Hara et al., 1984). The stimuli included L-methionine [Met, a neutral amino acid with long side-chains (LCN)], L-alanine [Ala, a neutral amino acid with short side-chain (SCN)], L-arginine (Arg, a basic amino acid), L-glutamic acid (Glu, an acidic amino acid), adenosine 5'-triphosphate (ATP, a nucleotide), and a mixture of bile salts (MBS, sodium salts of cholic acid, taurocholic acid and taurolithocholic acid). With the exception of the daily preparation of ATP at 10^{-3} M, stock solutions of individual stimuli (Sigma grade; Sigma Chemical Co., St. Louis, MO.) were prepared at 10^{-2} M weekly in charcoal-filtered artesian tap water (pH approximately 8.5) and stored at 4^{o}C. Charcoal-filtered artesian tap water was used to dilute the stock solutions during the experiments. Test concentrations were 10^{-4} M Met, Ala, Arg and ATP, $3x10^{-4}$ M MBS (10^{-4} M for each of the three components in the mixture) and 10^{-3} M Glu. The water control was obtained from the same charcoal-filtered tap water source as that used to prepare the stimulus solutions. The pH values of all individual solutions tested were between 8.2 and 8.8, the pH of both the natural water where the fish were obtained and the pH of the aquaria water.

Six different binary mixtures of amino acids were tested. The mixtures were formed by mixing equal aliquots of two amino acid

solutions at double the concentrations used for testing the individual components. Thus, the concentration of each component within a binary mixture was the same as that tested individually.

One ml of stimulus solution at room temperature was injected by a Pasteur pipette into a flow (12-14 ml/min) of charcoal-filtered artesian tap water which continuously bathed the olfactory mucosa (Caprio and Byrd, 1984). The manual sample injection valve used in the experiments described in chapters I, III and IV of this dissertation was not used for these experiments since switching the valve generated a pressure pulse which caused movement of the olfactory lamella adjacent to the recording electrode, thereby leading to recording instability. The test solutions were diluted by the stimulus delivery system, and the maximal concentration delivered to the olfactory organ was 42% of the original concentrations injected as determined by photodensitometry of dye solutions (Caprio and Byrd, 1984). All stimulus concentrations listed in the text are the injected concentrations. To minimize cross-contamination, the stimulus port was continuously flushed with the charcoal-filtered tap water during the 2-minute inter-stimulus intervals. Whenever possible, a stimulus solution was presented twice to each ORN recorded.

Recording Preparation

The olfactory lamellae were exposed by removing the skin, connective tissue and cartilage dorsal to the nasal capsule. Single unit activity, mostly greater than 400 μV in amplitude, was recorded extracellularly (bandpass 30-3000 Hz) from 69 ORNs with low-impedance

(60-200 KΩ), metal-filled glass microelectrodes, tip-plated with platinum-black (tip diameter ranging from 4-8 μm) (Gesteland, 1975). Using a micromanipulator and hydraulic microdrive (Narishige MO-8), an electrode was placed against the sensory area of a lamella (Caprio and Raderman-Little, 1978; Erickson and Caprio, 1984) to search for spontaneous single unit activity. ORNs recorded satisfied the single unit criteria of interspike intervals greater than the relative refractory period (approximate 4 ms) and a similarity among action potential waveforms (Baylin, 1979; Revial et al., 1982). The position of the recording electrode was adjusted whenever necessary to optimize the signal-to-noise ratio. Only those recordings which lasted ≥10 minutes were included in the present report. The action potential activity of ORNs, which included at least 15 seconds of prestimulus and 30 seconds of response time for each trial, was amplified, digitized (Neuro-corder, Neuro Data Instruments Corp., New York) and stored on one video channel of a VHS video recorder. The simultaneously recorded underwater electro-olfactogram (EOG), a slow negative potential change in the water obtained immediately above the olfactory mucosa in response to chemical stimulation, was detected with calomel electrodes via Ringer-agar-filled capillary pipettes (Silver et al., 1976; Kang and Caprio, 1991). The EOG signals were amplified by a direct-coupled amplifier, displayed on an oscilloscope and stored on a second video channel of the VCR recorder. A voice description of the experimental procedure was recorded on an audio channel of the video recorder.

Data Acquisition, the Response Measure and Analysis

Data were analyzed off-line. Action potentials were digitized at 32 KHz with a computerized data acquisition and analysis system (BrainWave Systems Discovery package, DataWave Technologies Corporation, Longmont, CO), which allowed all single-unit discrimination to be performed in software. Spike events, EOG signals and experimental parameters, i.e., beginning of a recording period, onset of stimulation and end of the recording period, were time-stamped with a 32 bit 100 μs resolution value and were saved in the data file. The BrainWave data files were displayed on a computer screen and printed out for initial visual analysis. For statistical analyses, the data files were exported as ASCII files for counting action potentials using BASIC-language programs developed by Dr. Rainer Voigt (Boston University Marine Program, Woods Hole, MA).

The numbers of action potentials of single ORNs occurring within successive 200-ms time bins during 5-s prestimulation and 5-s stimulation periods for each trial and for water controls were subjected to the interrupted time-series analysis (Hudson, 1977). The interrupted time-series test determined the effectiveness of a treatment that is conducted singly, but in a time-series design. The significance of a response to a stimulation was determined by comparing the obtained t value with the tabled critical t value (α=0.05) (Hudson, 1977). Responses of single ORNs to stimuli were classified as excitatory (E), suppressive (S), and null (N) responses according to the statistical analysis. Although "null" literally

means "no significant change" from spontaneous activity, for the sake of simplicity in descriptions of the present experiments "null" is defined as a response type (i.e., a "no response").

The relationship between overall responses to pairs of odorant stimuli across the ORNs sampled was examined by multivariate analysis of variance (MANOVA). The number of action potentials occurring within successive 500-ms time bins for the 5-s response periods across the units were subjected to MANOVA tests using SAS (1986, SAS Institute Inc., Cary, NC). Wilk's Lambda was used to test MANOVA significance.

RESULTS

Recording Time and Spontaneous Activity

The responses of 69 spontaneously active ORNs obtained from 22 catfish were studied (Table II.1). Recording time ranged from 10 to 72 minutes per receptor cell with an average of 24±15 minutes/cell (mean±SD). The spontaneous frequency ranged from <1 to 12.3 action potentials/second with a mean frequency of 4.7±2.5 (mean±SD) action potentials/second (Fig. II.1).

Olfactory Receptor Responses to Single Odorants

Based on interrupted time-series analysis, responses of single ORNs were classified as excitatory (E), suppressive (S), or null (N) (Figs. II.2 and II.3; Table II.1). For 37 ORNs that were each tested with the four amino acids (experiment A, *neurons* #12-48, Tables II.1 and II.2), 10 ORNs (27%) responded (with excitation or suppression) to one, 14 ORNs (38%) to two, four ORNs (11%) to three, and one ORN (3%) to four amino acids; eight ORNs (22%) failed to respond to any of the

Table II.1. *Responses* of Single Olfactory Receptor Neurons to Different Stimuli*

ORN#	Met	Ala	Arg	Glu
EXPERIMENT A**				
1	S	S	S	-
2	S	S	S	-
3	S	-	S	S
4	S	-	N	S
5	S	-	N	N
6	S	-	N	N
7	N	-	E	E
8	N	-	S	N
9	N	-	N	N
10	-	E	E	E
11	-	E	N	E
12	E	E	N	E
13	E	N	E	N
14	E	N	N	N
15	S	S	S	S
16	S	S	N	S
17	S	S	N	S
18	S	S	N	N
19	S	N	N	N
20	S	N	N	N
21	N	E	E	N
22	N	E	N	N
23	N	S	E	N
24	N	S	E	N
25	N	S	S	S
26	N	S	S	N
27	N	S	N	E
28	N	S	N	S
29	N	S	N	S
30	N	S	N	N
31	N	N	E	E
32	N	N	E	E
33	N	N	E	S
34	N	N	S	E
35	N	N	S	S
36	N	N	S	N
37	N	N	S	N
38	N	N	S	N
39	N	N	N	E
40	N	N	N	E
41	N	N	N	N
42	N	N	N	N
43	N	N	N	N
44	N	N	N	N
45	N	N	N	N
46	N	N	N	N
47	N	N	N	N
48	N	N	N	N

(table *con'd.*)

ORN#	Met	Ala	Arg	Glu	MBS	ATP
EXPERIMENT B***						
49	S	N	N	-	E	S
50	N	S	S	N	N	-
51	N	S	N	S	N	-
52	N	N	N	N	N	N
53	-	S	N	N	N	N
54	E	E	N	S	N	N
55	E	S	N	N	N	N
56	E	N	N	N	E	S
57	S	E	S	S	N	S
58	S	S	E	E	E	E
59	S	N	N	N	N	N
60	N	S	S	N	N	N
61	N	S	N	N	S	S
62	N	N	S	N	E	N
63	N	N	S	N	S	N
64	N	N	N	N	S	N
65	N	N	N	N	S	N
66	N	N	N	N	N	N
67	N	N	N	N	N	N
68	N	N	N	N	N	N
69	N	N	N	N	N	-

* E, S, and N indicate excitatory, suppressive, and null responses, respectively. "-" indicates no tests conducted. Test concentrations were 10^{-4} M Met, Ala, Arg and ATP; $3x10^{-4}$ M MBS (10^{-4} M each for sodium salts of cholic acid, taurocholic acid and taurolithocholic acid); and 10^{-3} M Glu. Neurons are arbitrarily numbered in accordance with the number of stimuli applied and for ease of comparison across neurons.

** Experiment A, ORNs tested with 3-4 amino acids.

*** Experiment B, ORNs tested with 3-4 amino acids and MBS and/or ATP.

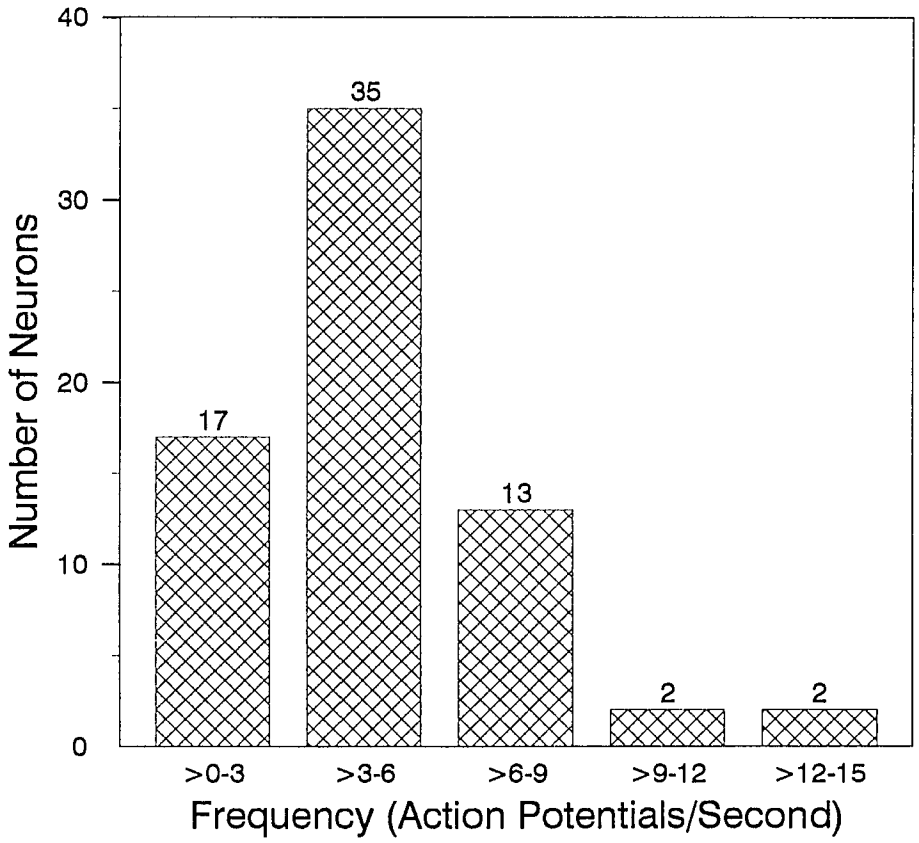


Fig. II.1. Frequency distribution of the spontaneous activity of the 69 recorded ORNs.

Fig. II.2. Simultaneous recordings of the electro-olfactogram (EOG) from the channel catfish olfactory epithelium (single upper traces; negative downward) and action potentials from single ORNs (dual lower traces) showing the three possible "response" types. (*A*) Duplicate excitatory receptor responses to $3x10^{-4}$ M MBS (mixture of bile salts; *neuron* #62); (*B*) Duplicate suppressive receptor responses to 10^{-4} M Met (*neuron* #1); (*C*) Duplicate null activity (no significant change from spontaneous activity) to 10^{-4} M Met (*neuron* #44). The onset of the EOG response was used to estimate the onset of the receptor responses. Based on the EOG responses, the action potential record was divided into two periods by the dotted lines: period I, prestimulation period and period II, stimulation period. Responses of ORNs to a stimulus were classified as either excitatory (E), suppressive (S), or null (N) based on the interrupted time-series analysis of the numbers of action potentials occurring within 200-ms time bins during 5-s prestimulation (Period I) and 5-s stimulation (Period II) periods.

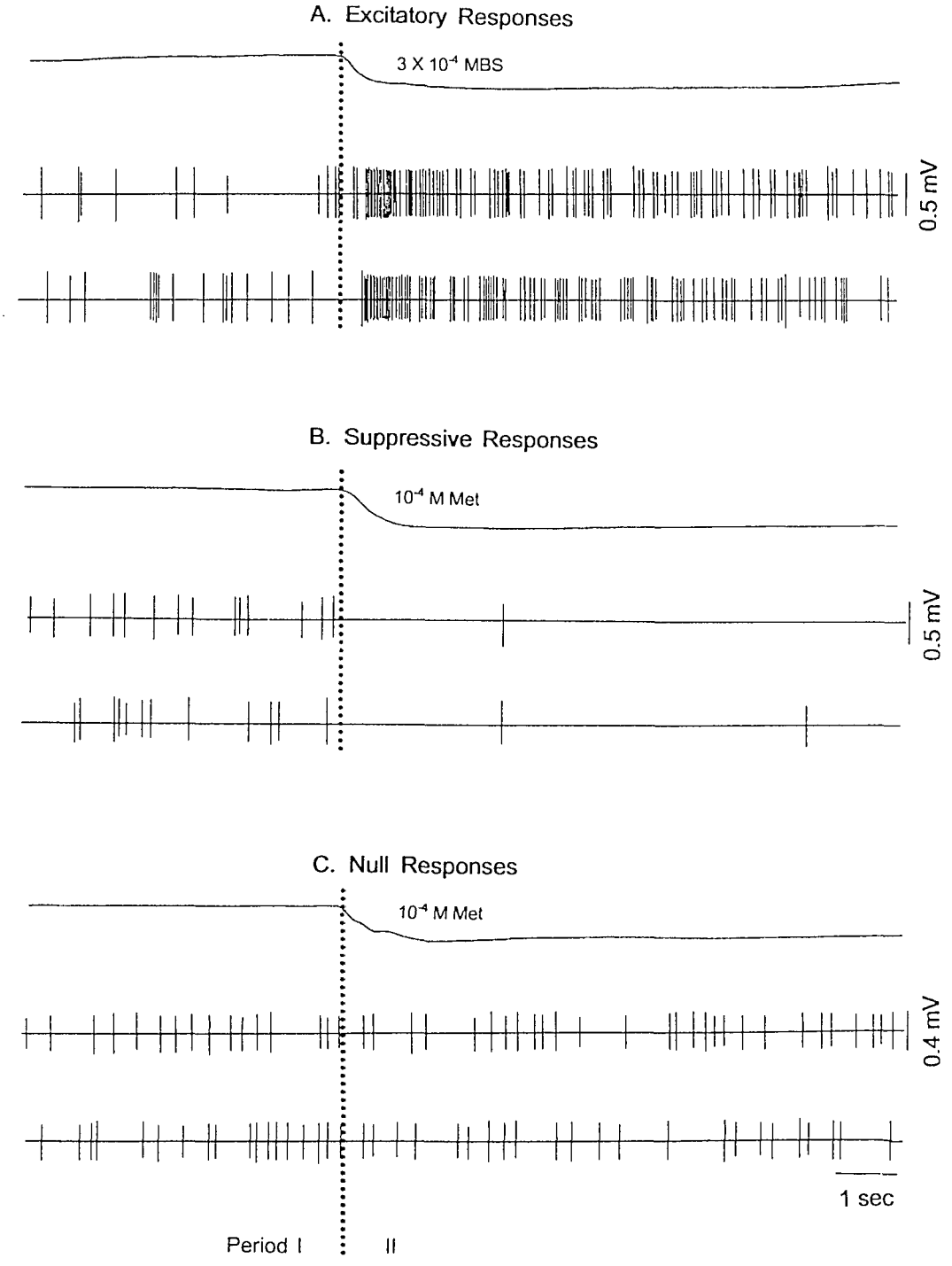
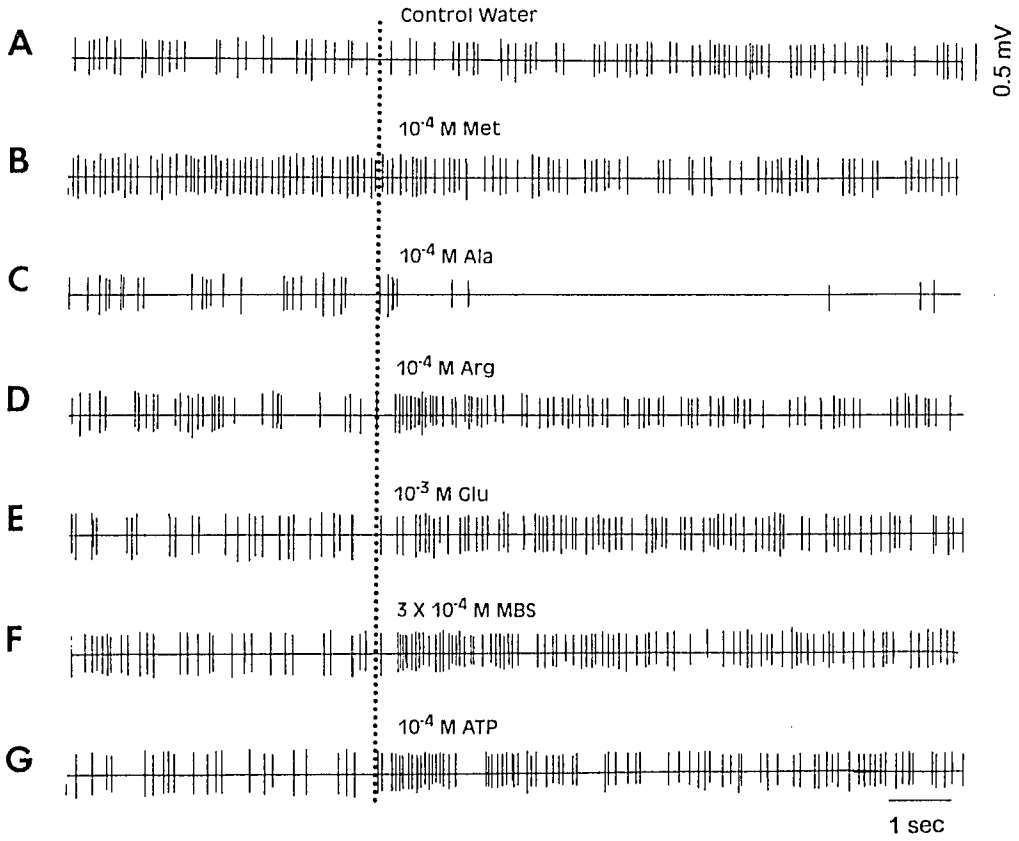

A. Excitatory Responses
3 X 10⁻⁴ MBS
0.5 mV
B. Suppressive Responses
10⁻⁴ M Met
0.5 mV
C. Null Responses
10⁻⁴ M Met
0.4 mV
1 sec
Period I
II

Fig. II.3. Representative responses of a single ORN (*neuron* #58) to six stimuli and a water control. (A) null activity to control water. (B) suppressive response to 10^{-4} M Met; (C) suppressive response to 10^{-4} M Ala; (D) excitatory response to 10^{-4} M Arg; (E) excitatory response to 10^{-3} M Glu; (F) excitatory response to $3x10^{-4}$ M MBS (sodium salts of cholic acid, taurocholic acid and taurolithocholic acid each at 10^{-4} M); and (G) excitatory response to 10^{-4} M ATP. The onset of the neural responses was indicated by a dotted line which was determined by the onset of the EOG responses (not shown). With the exception of Met, all stimuli were tested on this receptor cell at least twice, and similar response patterns to each stimulus repetition (not shown) were observed.


A
Control Water
0.5 mV
B
10^{-4} M Met
C
10^{-4} M Ala
D
10^{-4} M Arg
E
10^{-3} M Glu
F
3 X 10^{-4} M MBS
G
10^{-4} M ATP
1 sec

amino acids tested. For 21 ORNs that were tested with the previous amino acids and ATP and/or MBS (experiment B, *neurons* #49-69, Tables II.1 and II.2), four ORNs (19%) responded (i.e. with excitation or suppression) to only one, six ORNs (29%) to two, four ORNs (19%) to three, and single ORNs (5%) to five and six stimuli, respectively; none of the ORNs responded to four stimuli and five ORNs (24%) failed to respond to any of the amino acids tested. No obvious patterns of responses were observed for individual ORNs based on stimulus quality.

Of the 45 ORNs which were tested with 4-6 stimuli and responded with excitation or suppression to at least one stimulus (Table II.2), four were excited and ten were suppressed by only a single stimulus. For this limited sample, only 31.1% were "narrowly-tuned" to a single stimulus. No ORNs recorded responded excitedly to all the stimuli (4-6) presented, but one neuron (*neuron* #15, Table II.1) was significantly suppressed by the four amino acids (Fig. II.4; Table II.2).

Different response types were elicited from the same ORN by different stimuli (Figs. II.3 and II.4; Table II.1). Chi-square analysis indicated that the responses to pairs of stimuli across the units were not significantly correlated (X^2=25.94, df=28, P>0.05; Table II.3). Multivariate analysis (MANOVA tests) conducted on the number of action potentials occurring within successive 500-ms time bins for 5-s response periods across the units also indicated that the overall responses of ORNs to pairs of stimuli were not significantly correlated (P>0.05, Table II.3).

Table II.2. *Response distribution of Olfactory Receptor Neurons*

Number of Stimuli Eliciting Responses*	Number of Stimuli Applied				Total
	Experiment A		Experiment B		
	3	4	5	6	
0	1	8	1	4	14
1	3	10	1	3	17
2	3	14	2	4	23
3	4	4	1	3	12
4		1	0	0	1
5			0	1	1
6				1	1

*Responses include both excitation and suppression.

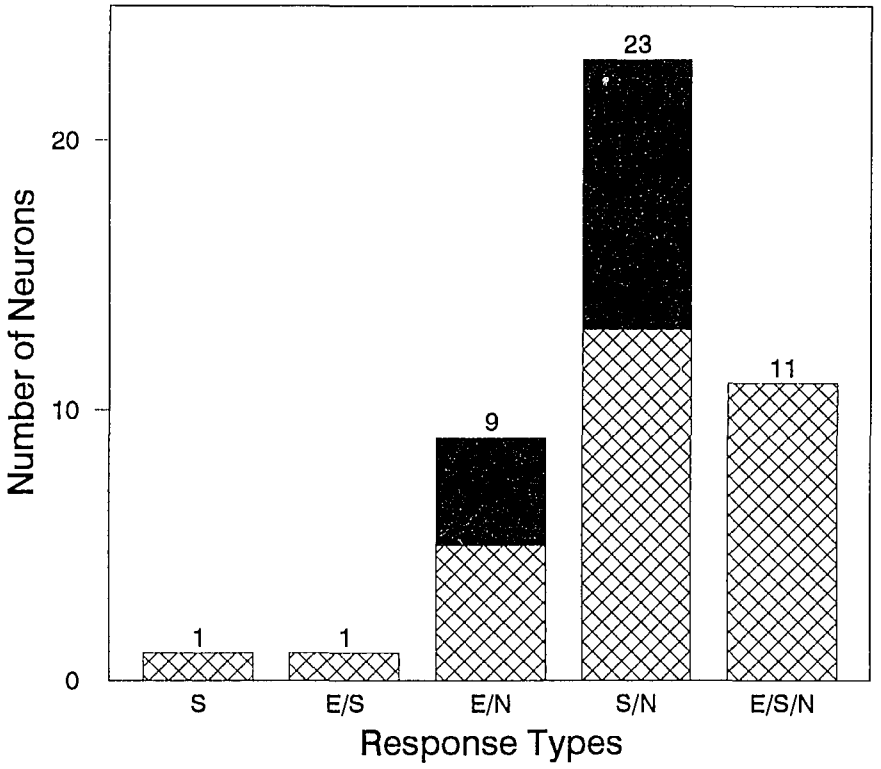


Fig. II.4. Distribution of response types of single ORNs to 4-6 stimuli. The interrupted time-series analysis classified neural activities into excitatory (E), suppressive (S), and null (N) responses. S: suppressive response; E/S: excitatory or suppressive response; E/N: excitatory or null response; S/N: suppressive or null response; E/S/N: excitatory, suppressive or null response. Black bars in E/N and S/N represent the ORNs which were excited (E/N) or suppressed (S/N) by only one of the 4-6 stimuli presented.

Table II.3. *Responsiveness* of Olfactory Receptor Neurons to Stimulus Pairs*

Stimuli		Both A & B		A,	B,	Neither	MANOVA**	Total
A	B	Same	Diff.	Not B	Not A	A nor B	(*P* Value)	Units
Met	Ala	9(15)	2(3)	7(12)	14(24)	27(46)	.1147	59
Met	Arg	6(9)	1(1)	15(23)	19(29)	25(38)	.1287	66
Met	Glu	7(11)	2(3)	10(16)	13(21)	31(49)	.5563	63
Ala	Arg	9(15)	4(6)	15(24)	11(18)	23(37)	.7457	62
Ala	Glu	10(17)	4(7)	12(20)	7(12)	26(44)	.2566	59
Arg	Glu	10(15)	2(3)	13(20)	12(18)	29(44)	.4716	66
Met	MBS	1(5)	2(10)	4(20)	5(25)	8(40)	.2850	20
Met	ATP	2(12)	2(12)	3(17)	1(6)	9(53)	.5749	17
Ala	MBS	1(5)	1(5)	7(33)	6(29)	6(29)	.5951	21
Ala	ATP	1(6)	2(11)	4(22)	2(11)	9(50)	.4856	18
Arg	MBS	2(10)	1(5)	3(14)	5(24)	10(47)	.3208	21
Arg	ATP	2(11)	0(0)	3(17)	3(17)	10(55)	.4026	18
Glu	MBS	1(5)	0(0)	3(15)	6(30)	10(50)	.5147	20
Glu	ATP	2(12)	0(0)	1(6)	2(12)	12(70)	.4662	17
MBS	ATP	2(11)	2(11)	4(22)	1(6)	9(50)	.6532	18

* Judged by interrupted time-series analysis. "Both A and B" denotes that both stimuli in the pair elicited statistically significant responses (i.e., either excitatory or suppressive responses) from ORNs (Same: the response types to each stimulus were the same; Different: the response types to each stimulus were different); "A, not B" denotes that only stimulus A in the pair elicited statistically significant responses; "B, not A" denotes that only stimulus B elicited statistically significantly responses; and "Neither A nor B" denotes that both stimuli in the pair failed to elicit statistically significant responses. Numbers in each column indicate the unit number in that category and the percentages (in parentheses) of units relative to the total unit number tested. See Table II.1 for definition of MBS.

** MANOVA were conducted on responses to all pairs of amino acids across the units tested.

Each stimulus elicited all three types of responses (i.e., E, S, and N) across the different ORNs recorded (Fig. II.5; Table II.1). Overall, out of a total of 303 stimulus presentations, 12.9% of the responses were classified as excitatory [39±18 (mean±SD) action potentials/5 second], 24.8% as suppressive [15±12 (mean±SD) action potentials/5 second] and 62.3% as null [23±14 (mean±SD) action potentials/5 second]. Chi-square analysis conducted on the number of ORNs grouped according to response types and the stimuli administrated indicated there was no significant relationship (X^2=7.26, df=10, P>0.05) between any particular response type and any specific stimulus.

Olfactory Receptor Responses to Binary Mixtures of Amino Acids

Responses to 6 different binary mixtures of amino acids and to their components were recorded from 19 single ORNs (Table II.4). The responses were classified as excitatory (E), suppressive (S), or null (N) based on the interrupted time-series analysis. For binary mixtures whose component amino acids both evoked suppressive responses from ORNs (n=6; Table II.4), the response to the corresponding mixture was suppressive (Figs. II.6*A* and II.7). When component amino acids of binary mixtures failed to produce a significant response from ORNs (n=20; Table II.4), the mixtures also failed to elicit significant responses (Fig. II.7). No mixtures were tested in which both component amino acids were excitatory.

Binary mixtures whose components elicited different response types generally elicited the response type of one of the components. The majority of the ORNs (8 of 9; 88.2%) responded with excitation (E)

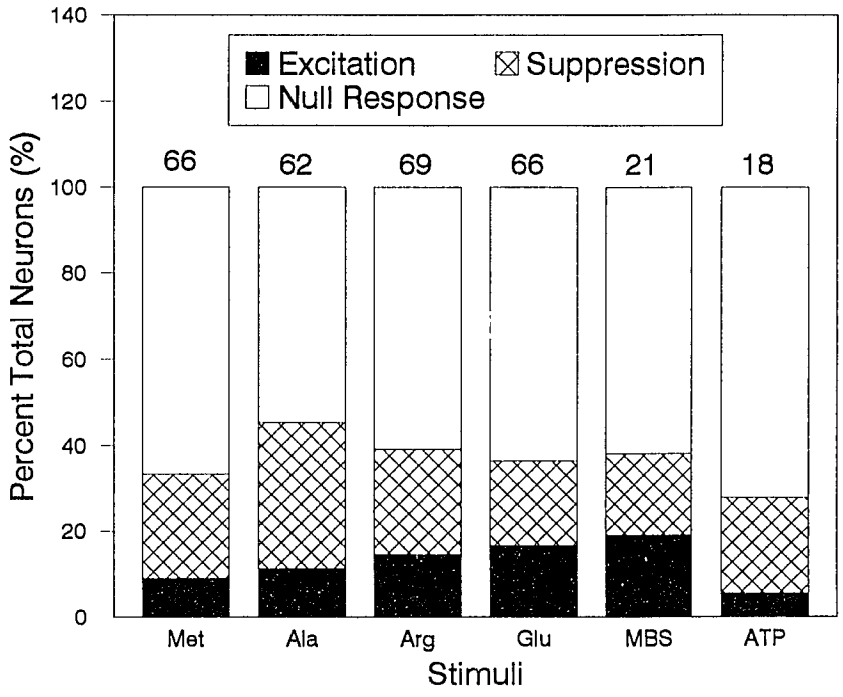


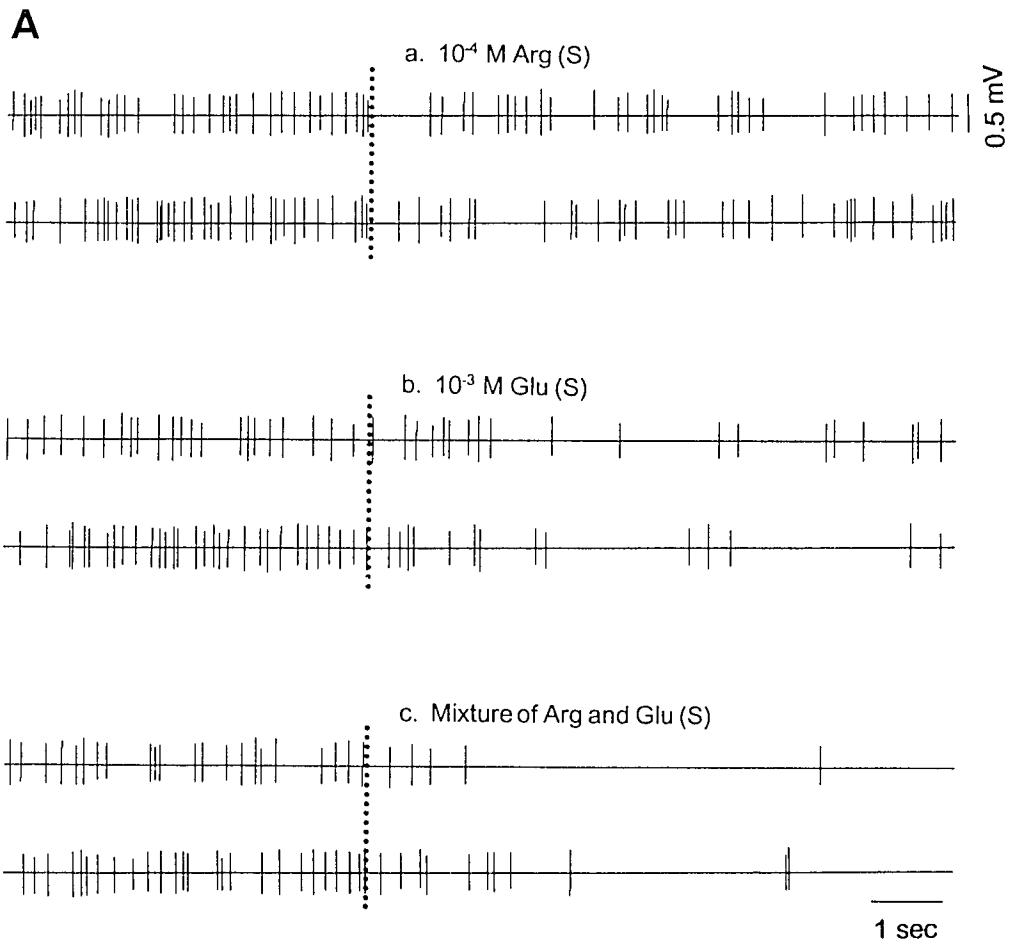
Fig. II.5. Distribution of E, S and N response types to each stimulus tested across the sampled ORNs. Numbers above each bar are the total number of ORNs tested with the corresponding stimulus.

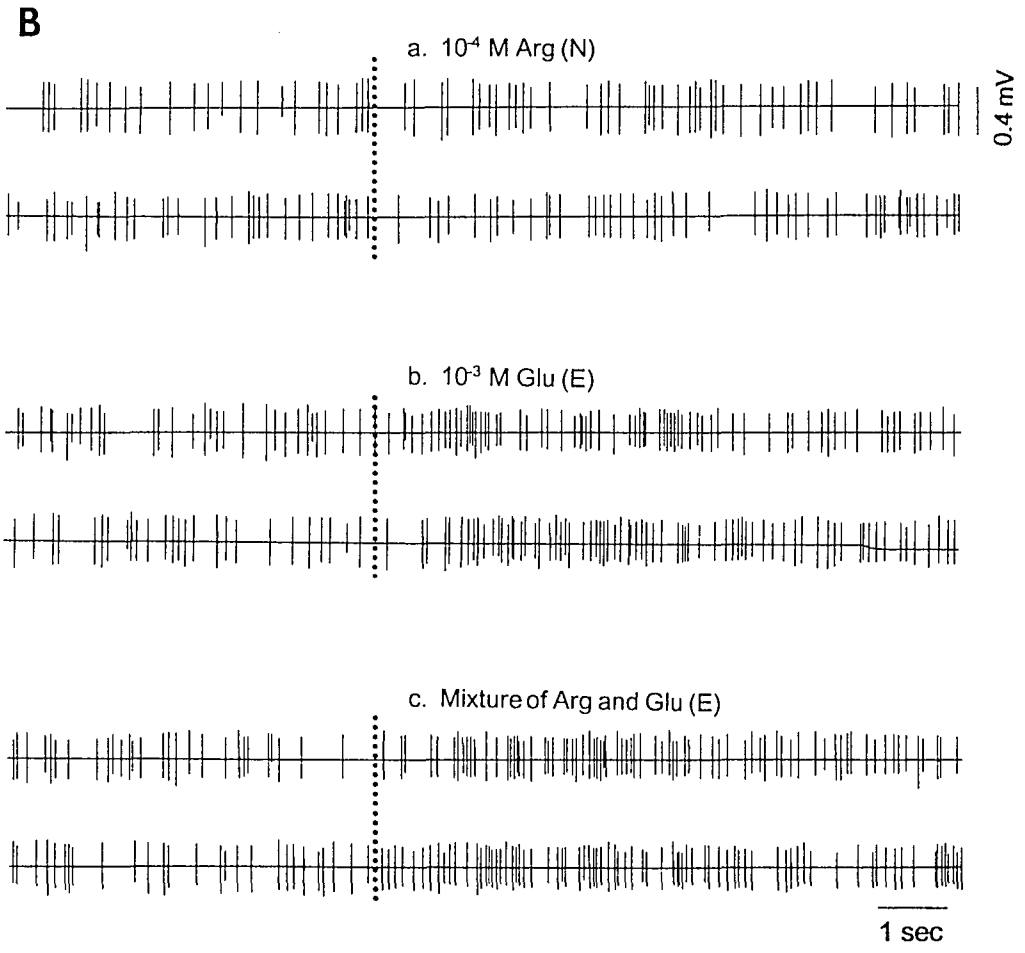
Table II.4. *Responses to Binary Mixtures and to Their Component Amino Acids*

Cell Number	Response to Mixtures		Response Types to Components	
	# of tests	Types	AA *a**	AA *b**
1. Met+Ala				
14	1	E	E	N
15	2	S	S	S
20	2	S	S	N
38	2	N	N	N
40	2	N	N	N
41	2	N	N	N
43	2	N	N	N
61	1	S	N	S
2. Met+Arg				
20	2	N	S	N
33	2	E	N	E
34	2	N	N	S
37	1	S	N	S
40	2	N	N	N
41	2	N	N	N
47	1	N	N	N
3. Met+Glu				
15	2	S	S	S
28	2	N	N	S
34	2	E	N	E
38	2	N	N	N
39	2	E	N	E
47	2	N	N	N
61	2	N	N	N
4. Ala+Arg				
15	2	S	S	S
20	1	N	N	N
33	2	E	N	E
38	2	N	N	S
40	2	N	N	N
47	2	N	N	N
63	1	S	N	S
5. Ala+Glu				
39	2	E	N	E
40	2	N	N	E
41	2	N	N	N
44	1	N	N	N
6. Arg+Glu				
3	2	S	S	S
14	2	N	N	N
15	2	S	S	S
27	2	E	N	E
38	2	N	S	N
39	2	E	N	E
41	2	N	N	N
43	2	N	N	N
47	2	N	N	N
57	1	S	S	S
61	2	N	N	N

* AA *a* and *b* refer to the first and second amino acids, respectively, in each of the corresponding mixtures.

Fig. II.6. Examples of responses of single ORNs to binary mixtures and to their components. (*A*) S+S mixtures (*neuron* #3). (*a*) Duplicate suppressive responses to Arg. (*b*). Duplicate suppressive responses to Glu. (*c*) Duplicate suppressive responses to binary mixtures of Arg and Glu. Concentration of Arg and Glu in the binary mixture was equal to the concentrations tested individually in *a* and *b*. (*B*) E+N mixtures (*neuron* #27). (*a*) Duplicate null responses to Arg. (*b*). Duplicate excitatory responses to Glu. (*c*) Duplicate excitatory responses to binary mixtures of Arg and Glu. Concentration of Arg and Glu in the binary mixture was equal to the concentrations tested individually in *a* and *b*. Dotted lines indicate the onset of the responses based on the onset of the EOG responses (not shown).


A
a. 10^{-4} M Arg (S)
0.5 mV
b. 10^{-3} M Glu (S)
c. Mixture of Arg and Glu (S)
1 sec


B
a. 10^{-4} M Arg (N)
0.4 mV
b. 10^{-3} M Glu (E)
c. Mixture of Arg and Glu (E)
1 sec

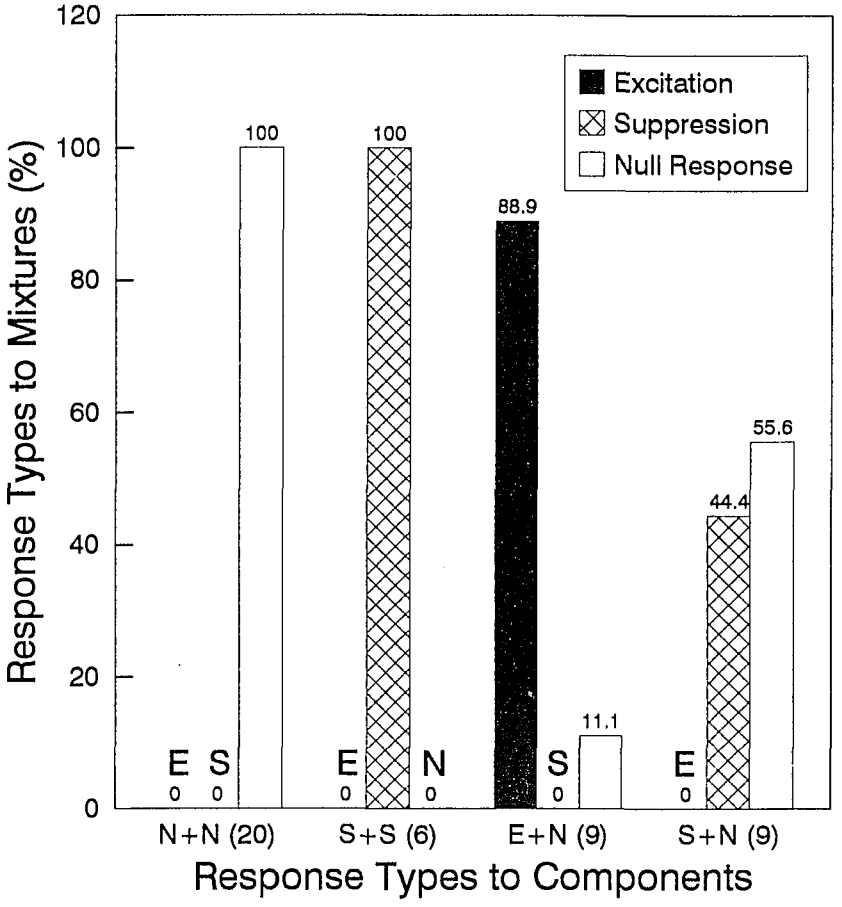


Fig. II.7. Summary of responses of single ORNs to binary mixtures of amino acids. N+N: Mixtures whose components failed to result in significant responses; S+S: Mixtures whose components elicited suppressive responses; S+N: Mixtures whose components elicited suppressive and null responses, respectively; E+N: Mixtures whose components elicited excitatory and null responses, respectively. Numbers in parentheses represent the number of cells.

to different binary mixtures whose components resulted in E and N types of responses (i.e., E+N mixtures), whereas only one ORN (11.1%) resulted in null (N) activity (Table II.4; Figs. II.6*B* and II.7). For binary mixtures whose components resulted in S and N types of responses, four ORNs (44.4%) responded with suppression (S) and five (55.6%) with null activity (Table II.4; Fig. II.7). No mixtures were tested in which one of the component amino acid was excitatory and the other was suppressive.

DISCUSSION

Spontaneous Activity

Due to the search strategy, all ORNs studied in the present report were spontaneously active. The average spontaneous frequency of 4.7±2.5 (mean±SD) action potentials/second was greater than <1 action potential/second characteristically observed in amphibian ORNs (O'Connell and Mozell, 1969; Getchell, 1973; Getchell, 1974; Duchamp et al., 1974; Blank and Mozell, 1976; Revial et al., 1978a; Revial et al., 1982; Baylin, 1979; Sicard and Holley, 1984; Holley et al., 1974; Getchell and Shepherd, 1978a). Possible reasons for the substantially greater spontaneous activity of channel catfish ORNs are species differences and/or a possible sampling bias due to the search strategy. The difference in spontaneous activity of ORNs between catfish and amphibians, however, parallels the finding of a greater spontaneous activity of catfish olfactory bulb neurons (chapter III) compared to that for amphibians.

ORN Response Specificities to Single Stimuli

Suppressive Responses. With the single exception in frogs (Blank and Mozell, 1976), previous *in vivo* extracellular recordings reported that suppressive responses of ORNs in amphibians to volatile stimuli were rare and were assumed not to be important for olfactory coding (Holley, 1991; Duchamp-Viret et al., 1989; Duchamp-Viret et al., 1990; Sicard, 1985; Sicard and Holley, 1984; Revial et al., 1982; Baylin, 1979; Revial et al., 1978a; Revial et al., 1983; Holley et al., 1974; Duchamp et al., 1974; O'Connell and Mozell, 1969; Gesteland et al., 1963; Getchell and Shepherd, 1978a; Getchell and Shepherd, 1978b). However, suppressive responses of ORNs were previously reported for insects (Akers, 1992; Sun et al., 1993; De Jong and Visser, 1988; Boeckh, 1976; Den Otter, 1977) and for aquatic animals, e.g., lobster (McClintock and Ache, 1989; Michel et al., 1991; Michel and Ache, 1994), lamprey (Suzuki, 1982), catfish (Ivanova and Caprio, 1993), and mudpuppy (Dionne, 1992). Furthermore, it was demonstrated that excitatory and suppressive olfactory receptor responses of beetle (Boeckh, 1976) and lobster (Michel et al., 1991), respectively, were associated with depolarizing and hyperpolarizing receptor potentials. The present results (Figs. II.3 and II.6; Tables II.1 and II.4) strongly suggest that suppressive responses of ORNs in channel catfish play important roles in olfactory coding. The low incidence of suppressive responses reported in frogs and land-phase salamanders could possibly have been due to difficulties in determining suppressive responses from the reported low spontaneous rates. The

obvious advantage for receptor neurons possessing high spontaneous activity is that this activity can be modulated, either enhanced or suppressed, more effectively than those receptor neurons having a low spontaneous activity (Duchamp, 1982). Therefore ORNs with at least a moderate rate of spontaneous activity can encode more stimulus information than those neurons with a low spontaneous firing rate. The high spontaneous activity of ORNs is also advantageous to the investigator as it allows for an easy recognition of suppressive responses (Kauer, 1974).

Although the cellular mechanisms underlying the suppressive responses is unknown, studies on isolated ORNs of salmon (Nevitt and Moody, 1992), channel catfish (Ivanova and Caprio, 1993; Miyamoto et al., 1992) and mudpuppy (Dionne, 1992) suggested that ORNs possess multiple transduction pathways for olfactory signal processing. Furthermore, it was observed from isolated ORNs in both vertebrates (Dionne, 1992) and invertebrates (Michel et al., 1991) that hyperpolarizing potentials associated with suppressive responses appeared to arise from the activation of K^+ channels. In channel catfish, however, no hyperpolarizing potentials associated with suppressive responses induced directly by amino acids were observed from isolated ORNs, but the reported modulation by amino acids of voltage-gated K^+ channels was suggested to be a mechanism responsible for these suppressive responses (Ivanova and Caprio, 1993).

Independent Stimulus Binding Sites. Results from previous electrophysiological (Caprio and Byrd, 1984; Ohno et al., 1984; Sveinsson and Hara, 1990b; Sveinsson and Hara, 1990a) and biochemical

(Cagan and Zeiger, 1978; Cancalon, 1978; Novoselov et al., 1980; Brown and Hara, 1981; Rhein and Cagan, 1983; Rehnberg and Schreck, 1986; Kalinoski et al., 1987; Bruch and Rulli, 1988) investigations in different species of fishes indicated that multiple olfactory receptor sites exist for amino acids. For the channel catfish, both electrophysiological (Caprio and Byrd, 1984; Caprio et al., 1989; Kang and Caprio, 1991) and biochemical (Bruch and Rulli, 1988) studies confirmed the independence of olfactory receptor sites for acidic, basic and neutral amino acids. Further, evidence exists for the independence of receptor sites for bile salts (Hara et al., 1984) and ATP (Michel et al., 1988), respectively, from those for amino acids. The present results that no two stimuli produced identical response patterns across the ORNs sampled (Table II.3) support the previous report of the independence of olfactory receptor sites for different amino acids, bile salts and ATP in the channel catfish. However, the present results do not exclude the possibility that the olfactory receptor sites for different stimuli have overlapping specificities or that one type of receptor site can be activated by a number of different molecules having similar stereochemical structures (Imamura et al., 1992).

Existence of Multiple Receptor Sites/Cell. The present study clearly showed that 55% of the single ORNs sampled responded to more than one of the tested stimuli that were indicated to bind to different receptor sites (Fig. II.3; Tables II.1 and II.2), which is consistent with previous reports indicating that single ORNs responded to multiple stimuli (Gesteland et al., 1963; Shibuya and Shibuya,

1963; Gesteland et al., 1965; O'Connell and Mozell, 1969; Shibuya and Tucker, 1967; Getchell, 1973; Getchell, 1974; Duchamp et al., 1974; Holley et al., 1974; Holley and DØving, 1977; Blank and Mozell, 1976; Revial et al., 1978a; Revial et al., 1978b; Revial et al., 1982; Revial et al., 1983; Sicard and Holley, 1984; Sicard, 1985; Mathews, 1972). It is also evident that different transduction pathways occur in the same ORN since the same ORN in the channel catfish responded to different stimuli with either excitation or suppression (Fig. II.3; Tables II.1 and II.2). *In vivo* electrophysiological studies in both amphibians and channel catfish support the hypothesis that single ORNs express several receptor site types (see Reviews by Kauer, 1987 and Holley, 1991). A recent whole cell patch clamp study (Ivanova and Caprio, 1993) of responses of isolated ORNs in the channel catfish also showed that individual receptor neurons responded with inward currents to different amino acid stimuli that were indicated from electrophysiological cross adaptation (Caprio and Byrd, 1984) and receptor binding (Bruch and Rulli, 1988) studies to bind primarily to different receptor sites. These data collectively indicate that multiple receptor site types for different odorants occur within the plasma membranes of individual ORNs, suggesting that odors may be coded by the pattern of activity across the different ORNs responding (i.e., an "across-fiber" scheme; see section *Quality Coding* for discussion).

A recent molecular study in the channel catfish, using the *in situ* hybridization technique (Ngai et al., 1993b), reported that each ORN expressed only a small subset of distinct odorant receptor

molecules, suggesting that the brain may discriminate among odors by which neurons are activated (i.e., a "labelled line" scheme; see section *Quality Coding* for discussion). This hypothesis, however, is inconsistent with the present electrophysiological results in the channel catfish which indicated that multiple receptor sites are expressed on single ORNs. The *in situ* hybridization study tested four probes which were specific for four gene subfamilies, comprising of 10-16 genes (Ngai et al., 1993a). The result that only 4% of the total ORNs were detected by these probes implied that only 25-40% of the total population of ORNs in catfish epithelium would express all of the genes coding the olfactory receptors, since it was estimated that the catfish genome encoded about 100 different olfactory receptor genes (Ngai et al., 1993b). Thus, it was possible that the receptor probes did not identify all the ORNs in which the respective receptors were expressed. Additional receptor probes could possibly have identified portions of the other 96% of the ORNs resulting in a probable overlap of expressed receptors per single ORNs. Additionally, three of the four receptor probes were specific for gene subfamilies, but could not distinguish among different members within a subfamily. However, it is possible that different members of such a gene subfamily might code for receptors which could be specific for structurally different odorants, since previous studies in bacteria indicated that a single point mutation could affect amino acid receptor specificity (Yaghmai and Hazelbauer, 1992).

ORN Subfamilies Specific for Groups of Odorants? The molecular study by Ngai et al (1993) also suggested that ORNs of channel catfish

may be classified into different subfamilies which are specific for groups of structurally different odorants; e.g., one subfamily might be specific for amino acids (Ngai et al., 1993b). The present experiments suggest, however, that single ORNs are not specific for only one type of odorant since only three groups of structurally different chemical compounds (amino acids, nucleotides and bile salts) were tested in the present study and 41% (7 of 17) of the responding ORNs were responsive to representatives of at least two of the three groups of stimuli tested, and four ORNs (24%) were responsive to representatives of all three groups (Experiment B in Table II.1). It is reasonable to expect, based on the present data, that if additional types of water-soluble stimuli were tested, some of the ORNs recorded would have also responded to some of these compounds. Furthermore, although some overlap existed with other neurons, the response spectra of the majority of ORNs tested were different from each other (Table II.1), suggesting that each ORN is highly individualized.

Quality Coding. How ORNs code stimulus quality is unresolved. The present results that 31% of the sampled ORNs responded with either excitation or suppression to only one of the 4-6 stimuli applied (Table II.2) were consistent with the suggestion that particular stimulus qualities might be coded by which specific neurons are activated (i.e. a "labelled line" code) (Lancet, 1991; Kauer, 1991; Derby and Ache, 1984a; Derby and Atema, 1988; De Jong and Visser, 1988); however, the limited number of stimuli and the single stimulus concentration tested for each of the ORNs in the present study are insufficient to determine the degree of stimulus specificity that the

31% of the neurons represent. The 69% of the ORNs tested which were relatively "broadly-tuned" (and the response specificity would probably increase with an increase in the number of stimuli and the concentrations of tested stimuli) suggest that odor quality is coded by the pattern of activity elicited by the stimulus across a population of neurons (i.e. an "across-fiber" scheme) (Scholz et al., 1993; Kauer, 1991; Derby and Ache, 1984a; Derby et al., 1984; De Jong and Visser, 1988; Carr and Derby, 1986a).

Responses to Binary Mixtures of Amino acids

The question of whether neural information concerning stimulus mixtures is processed differently than that of individual odorants is critical for a better understanding of the olfactory process. Numerous studies, especially those involving crustaceans, indicated the difficulty due to mixture interactions in predicting the responses to stimulus mixtures based on the responses to the individual components of the mixtures (Derby and Ache, 1984b; Zimmer-Faust et al., 1984; Derby et al., 1985; Gleeson and Ache, 1985; Johnson et al., 1985; Johnson et al., 1989; Borroni et al., 1986; Atema et al., 1989; Carr and Derby, 1986b; Carr and Derby, 1986a; Derby et al., 1991b; Derby et al., 1991a). In contrast, EOG and integrated multiunit responses of channel catfish ORNs to binary, trinary (Caprio et al., 1989) and more complex (Kang and Caprio, 1991) mixtures of amino acids were experimentally predictable with knowledge of both the responses to the individual components and the relative independence of the respective receptor sites. In the present report, no single response of ORNs to binary mixtures was classified differently from the

response to at least one of the components (Fig. II.7). The results that ORN responses to N+N and S+S mixtures were similar to those to their components (Figs. II.6A and II.7) indicated that mixture interactions in channel catfish were not common for the tested component-similar binary mixtures; however, additional tests are needed to determine whether the responses to E+E binary mixtures are also predictable. The present results are similar to those obtained from olfactory bulb neurons in the same species, indicating that it was generally possible to predict the response types to binary mixtures whose components evoked the same response types (Chapter IV).

For the majority (89%) of single ORNs tested with E+N binary mixtures, mixture interactions were rare and E responses were elicited (Figs. II.6B and II.7); however, for the S+N binary mixtures, null activity occurred in 56% of single ORNs recorded (Fig. II.7), indicating that a "null" component can sometimes mask the responses of a S or even an E (11%) component in a binary mixture. A similar result for E+N binary mixtures was reported for ORNs of the Colorado potato beetle (De Jong and Visser, 1988). Since component amino acids in the binary mixtures tested in the present study were suggested to bind primarily to different receptor sites, but having some overlapping specificities to other related sites (Kang and Caprio, 1991), the masking effects of the N components on the S components might have resulted from either a competitive (Michel et al., 1991; Gleeson and Ache, 1985; Ache et al., 1988; McClintock and Ache, 1989; Ache, 1989; Derby et al., 1991b) or a noncompetitive (Laing and Glemarec, 1992; Bell et al., 1987) mechanism of inhibition. For

example, for single olfactory bulb neurons in the channel catfish, the mean percent response change for the S components whose S+N mixtures elicited suppressive responses was significantly greater (i.e., more suppressed) than that caused by the S components whose S+N mixtures resulted in N responses (Table IV.2, Chapter IV). The sample size (n=9) for the S+N mixtures tested herein was, however, too small for a similar analysis as performed for single olfactory bulb neurons. Additional experiments with S+N mixtures, along with E+S mixtures, are necessary for a more complete understanding of the effects of stimulus mixtures at the receptor cell level. The present results, however, indicate that across the different types of binary mixtures tested, mixture interactions at the single ORN level were limited.

CHAPTER III

RESPONSES OF SINGLE OLFACTORY BULB NEURONS TO INDIVIDUAL AMINO ACIDS

INTRODUCTION

For a better understanding of olfaction and the coding of quality information in the olfactory system, it is critical to determine how single olfactory neurons respond to stimuli. The majority of information on single olfactory receptor neurons was derived from amphibians, and most of these results were obtained using stimuli of questionable biological relevance (see Kauer, 1987 for review). However, in fishes, water-soluble amino acids possibly serve as a source for conspecific recognition due to their presence in the mucus covering the external body of fishes (Hara et al., 1984; Uskova and Chaykovskaya, 1971; Stabell and Selset, 1980; Kotrschal, 1991), and for appetitive feeding behavior since they are released from both living aquatic vertebrates (Kleerekoper and Mogensen, 1959; Kleerekoper and Mogensen, 1963), invertebrates (Johannes and Webb, 1970) and decaying organic matter (see Caprio, 1988a, 1990 for reviews). Channel catfish have been used as an experimental model to investigate the physiology of the olfactory system in teleosts in numerous electrophysiological studies (Byrd and Caprio, 1982; Caprio, 1977; Caprio, 1978; Caprio, 1980; Caprio, 1988b; Caprio and Byrd, 1984; Caprio et al., 1989; Erickson and Caprio, 1984; Restrepo et al., 1990; Kang and Caprio, 1991; Miyamoto et al., 1992b; Miyamoto et al., 1992a; Ivanova and Caprio, 1993), biochemical studies (Restrepo et al., 1993; Restrepo and Boyle, 1991; Bruch and Rulli, 1988; Bruch and Kalinoski, 1987; Kalinoski et al., 1987; Cancalon, 1978) and molecular studies (Ngai et al., 1993b; Ngai et al., 1993a; Goulding et al., 1992). Due primarily to the small size and high density of the

olfactory receptor neurons, however, information is scant on the activity of single olfactory receptor neurons in intact preparations of any teleost. It is rather easy to record the neural activity of single mitral cells, the large output neurons of the olfactory bulb which receive, process and transmit odor information from olfactory receptor neurons to other parts of the brain (Shepherd, 1979). Thus, the present study was designed to characterize the response reproducibility, intensity-response functions, of olfactory bulb neurons in channel catfish, which were necessary for a subsequent investigation of the responses of olfactory bulb neurons to stimulus mixtures (Chapter IV).

Evidence from cross-adaptation (Caprio and Byrd, 1984) and biochemical binding (Bruch and Rulli, 1988) studies in channel catfish indicated the independence of olfactory receptor sites for acidic, basic and neutral amino acids. Recent electrophysiological studies on olfactory mixtures of amino acids provided additional evidence that the acidic, basic and neutral amino acids interacted with different receptor sites in the olfactory epithelium (Caprio et al., 1989; Kang and Caprio, 1991). It was unknown, however, whether the responses of single mitral cells in the olfactory bulb reflected different distributions of these receptor sites.

The present results indicate that: (a) responses of single olfactory bulb neurons to amino acids were highly reproducible over time, (b) responses of single olfactory bulb neurons to a given amino acid did not change from excitation to suppression, or *vice versa*, across different stimulus concentrations, (c) the changes in magnitude

of the responses of olfactory bulb neurons to increasing stimulus concentrations suggested that individual cells might discriminate stimulus intensity, and (d) with the sole exception of responses to L-methionine and L-norvaline, the overall responses to pairs of amino acids were not significantly correlated, indicating that the majority of amino acids tested were processed differently by the olfactory bulb.

METHODS AND MATERIALS

Animal Preparation

Forty-three channel catfish, *Ictalurus punctatus*, ranging from 16g to 70g, were obtained from nearby university ponds, were held in floating cages in the ponds and were fed with commercial catfish chow. Catfish brought into the laboratory's holding facility were maintained at approximately 25°C in aerated, charcoal-filtered water in 250-liter aquaria on a 12:12 light-dark regime, and were used experimentally within two weeks of laboratory holding time (Tucker, 1973).

Fish tested were immobilized with an intramuscular injection of Flaxedil (gallamine triethiodide; initially 0.05 mg/100g body weight), wrapped in wet tissue paper and held by orbital clamps in a Plexiglass container. Supplemental does of Flaxedil were applied to the fish as required. The anesthetic agent, MS-222, was not used due to its depression of both peripheral and central neural activity in fish (Spath and Schweickert, 1977; Meredith and Moulton, 1978). However, a local anesthetic, 3% tetracaine, was liberally applied to the skin surface prior to and during the surgery. Perfusion of aerated, charcoal-filtered tap (artesian) water over the gills at approximately

500 ml/min was accomplished by means of a forked teflon tubing which was placed beneath the operculi using a posterior approach throughout the experiment.

The olfactory lamellae were exposed by removing the skin, connective tissue, and cartilage dorsal to the nasal capsule. The olfactory bulb and tract of one side (either right or left) were exposed by the removal of the soft tissue and bone overlying them. A thin strip of skin and underlying tissue were left intact between the olfactory epithelium and bulb to prevent the stimulus flow to the olfactory mucosa from contacting the bulb.

Stimuli and Delivery

Stimuli were chosen from groups of L-amino acids known to interact with different olfactory receptor sites (Caprio and Byrd, 1984; Bruch and Rulli, 1988; Caprio et al., 1989; Kang and Caprio, 1991). The stimuli included neutral amino acids with long side-chains [L-methionine (Met) and L-norvaline (nVal)], neutral amino acids with short side-chains [L-alanine (Ala) and L-glutamine (Gln)], basic amino acids [L-arginine (Arg) and L-lysine (Lys)] and acidic amino acids [L-glutamic (Glu) and L-aspartic (Asp) acids]. Stock solutions of the individual amino acids (Sigma grade; Sigma Chemical Co., St. Louis, MO.) were prepared weekly in charcoal-filtered artesian tap water (pH approximately 8.5) at 10^{-2} M (except for Asp, 10^{-3} M) and stored at 4°C. On the day of the experiment, charcoal-filtered artesian tap water was used to dilute the stock solutions to the desired test concentrations. Except for determining intensity-response functions, all single amino acids were adjusted to the nearest whole log

concentration that elicited similar EOG magnitudes determined in previous experiments [i.e., 10^{-4} M for Met, nVal, Ala, Gln, Arg, and Lys and 10^{-3} M for Glu and Asp (Kang and Caprio, 1991)]. The pH values of all amino acid solutions tested remained between 8.0 and 8.6. Charcoal-filtered tap water continuously bathed the olfactory mucosa at a flow rate of approximately 8 ml/min.

Stimulus solutions were randomly introduced into a 2-ml Teflon loop of a manual sample injection valve (Omnifit USA Corp., Atlantic Beach, NY) and injected into the water bathing the olfactory organ. Photodensitometry studies indicated that the maximum stimulus concentration delivered to the olfactory mucosa was 75% of the concentration administered (values in the text are the undiluted values). The water control was taken from the same charcoal-filtered tap water source as that used to prepare the stimulus solutions. Interstimulus intervals were at least 2.5 min.

Olfactory Tract Stimulation

In a subset of experiments, electrical stimulation was used to confirm whether the recorded olfactory bulb neurons were most likely mitral cells. A total of 23 olfactory bulb neurons from four fish were subjected to electrical stimulation. After single-unit activity was encountered, constant-current stimulus pulses of 500-800 μs duration were delivered singly or in pairs to the ipsilateral olfactory tract. A concentric bipolar electrode with a tip diameter of approximately 600 μm (FHC, Brunswick, ME) was placed on the dorsal surface of the tract (diameter ranging from 550 to 700 μ) at positions 2-5 mm from the bulb. The current intensities ranged from 0.2 to 2.0

mA. The inter-stimulus intervals for single shocks and inter-train intervals for paired shocks were different from unit to unit, and generally ranged from 1 to 5 s. No collision tests were conducted due to the high frequency of spontaneous action potentials (see Results). Electrically evoked action potentials were registered by the recording electrode. The olfactory bulb neurons driven by electrical stimulations with short (<2 ms) and constant latencies were considered to be mitral cells (Meredith, 1986; Imamura et al., 1992).

Electrophysiological Recording Techniques

The underwater electro-olfactogram (EOG), a slow negative potential change in the water immediately above the olfactory mucosa in response to chemical stimulation, was recorded *in vivo* with calomel electrodes via Ringer-agar-filled capillary pipettes (Silver et al., 1976; Kang and Caprio, 1991). The EOG signals were amplified by a direct-coupled amplifier, displayed on an oscilloscope, digitized (Neuro-corder, DR-284s, Neuro Data Instruments Corp., New York) and stored on one video channel of a VCR recorder for subsequent analysis.

Unit activity (350-1200 μV peak-to-peak amplitude), was recorded extracellularly from 89 olfactory bulb neurons in 43 preparations with low-impedance (3-7 MOhm) glass micropipettes filled with 3 M NaCl. An electrode was slowly advanced vertically by a hydraulic microdrive into the olfactory bulb until a spontaneously active unit was encountered. The depth of the electrode tip within the olfactory bulb was measured from the calibrated hydraulic microdrive. The criteria for single units were the similarity among action potential waveforms and a minimum of 1-ms interspike intervals (Meredith and Moulton,

1978). The neural activity of single olfactory bulb neurons, which included at least 20 seconds of a prestimulation period and 40 seconds of a stimulation period, was amplified and stored on a second VCR video channel.

Data Acquisition and Analysis

Action potentials were digitized at 32 KHz with a computerized data acquisition and analysis system (BrainWave Systems Discovery package, DataWave Technologies Corporation, Longmont, CO), which allowed all single-unit discrimination to be performed with software. Spike events, EOG signals and experimental parameters, i.e., beginning of a recording period, onset of stimulation and end of the recording period, were time-stamped with a 32 bit 100 μs resolution value and were saved in a data file. The BrainWave data files were displayed on a computer screen and printed out for initial visual analysis. For statistical analyses, the data files were exported as ASCII files for action potential counting and inter-spike interval measuring using BASIC-language programs developed by Dr. Rainer Voigt (Boston University Marine Program, Woods Hole, MA). Since the slight pressure pulse caused by switching of the manual sample injection valve sometimes elicited brief changes in the spontaneous activity (see Fig. III.3), recordings for the 3-s period, prior to the onset of responses as judged from the EOG waveform, were not included in the analyses. Thus, statistical analyses described below were performed based on the numbers of action potentials occurring within certain time intervals in prestimulation and stimulation periods (i.e., period I and III, respective, in Fig. III.3).

To compare the response consistency of single cells to the same stimulus, a nonparametric statistical analysis (Spearman Correlation) was performed (Meredith and Moulton, 1978). The number of action potentials occurring within successive 1-s time bins over 10-s prestimulation and 20-s stimulation periods between two repeated trials of the same stimulus on the same cell were subjected to the Spearman Correlation tests using SAS (1986, SAS Institute Inc., Cary, NC). For comparison, Spearman correlations were also examined for responses of cells to randomly selected pairs of stimuli.

The responsiveness of olfactory bulb neurons to a stimulus was judged by one-tailed interrupted time-series analysis (Hudson, 1977). The interrupted time-series test determined the effectiveness of a treatment that is conducted singly, but in a time-series design. The numbers of action potentials of single olfactory bulb neurons occurring within successive 200-ms time bins during 5-s prestimulation and 5-s stimulation periods for each trial were subjected to the interrupted time-series analysis. The significance of a response to a stimulation was determined by comparing the obtained t value with the tabled critical t value (α=0.05) (Hudson, 1977). Responses of single olfactory bulb neurons to stimuli were classified as excitatory (E), suppressive (S), and null (N) according to the statistical analysis. Although "null" literally means "no significant change" from spontaneous activity, for the sake of simplicity in descriptions of the present experiments "null" is defined as a response type (i.e., a

"no response"). The responsiveness of cells to a water control was also examined by the interrupted time-series analysis.

The relationship between overall responses to pairs of odorant stimuli across the olfactory bulb neurons sampled was examined by MANOVA (multivariate analysis of variance). The number of action potentials occurring within successive 500-ms time bins for 5-s response periods across the units were subjected to MANOVA tests using SAS. Wilk's Lambda was used to test MANOVA significance.

Histology

Histological examinations were performed to determine how the mitral cells, the output neurons of olfactory bulbs in the channel catfish, distribute within the bulb. Six fish were fixed *in situ* with 4% paraformaldehyde. After the fixation, small crystals of diI (carbocyanine dye 1,1'diocadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) (Finger and Böttger, 1990) were applied to both olfactory tracts. The preparations with the tracts covered with agar were placed in paraformaldehyde solution in a 37^{o}C oven for 35-40 days. After this diffusion period, the olfactory bulbs were removed by carefully isolating them from the rest of the tissue and were embedded in egg yolk overnight in 4% paraformaldehyde solution. Horizontal or coronal sections at 30-50 μm were cut with a vibratome and mounted on microscope subbed slides. Sections were viewed by means of a compound Zeiss microscope equipped for epifluorescence with filter set number 487714.

RESULTS

Back-filling with diI indicated that the cell bodies of the mitral cells occurred in a concentric layer extending from the surface to a depth of approximately 200 μm (Fig. III.1A). The cell bodies of the mitral cells have various forms, such as oval, triangular, elongated, or fusiform and ranged from 13-31 μm by 25-48 μm (Fig. III.1A).

The responses of 89 single olfactory bulb neurons to amino acids, in which recordings lasted for at least 16 min (ranging from 16 to 344 min) per cell, were examined in 43 catfish. Most of the cells recorded were located in the mid-caudal regions of the olfactory bulb and at primarily two depths, 150-250 μm and 750-950 μm (Fig. III.1B)

All 89 cells recorded in this study were spontaneously active. The spontaneous frequency ranged from <1 to 15.9 action potentials/second with a mean frequency of 5.2 ± 3.6 (mean ± SD) action potentials/second (Fig. III.2). Amino acid stimulation resulted in excitatory (E), suppressive (S), or null (N) activity (Fig. III.3) based on interrupted time-series analysis.

In 16 of 23 cells tested in the electrical stimulation experiments (Fig. III.4), stimulation of the olfactory tract elicited action potentials with a short, consistent latency, providing evidence that the cells were most likely mitral cells.

Response Reproducibility

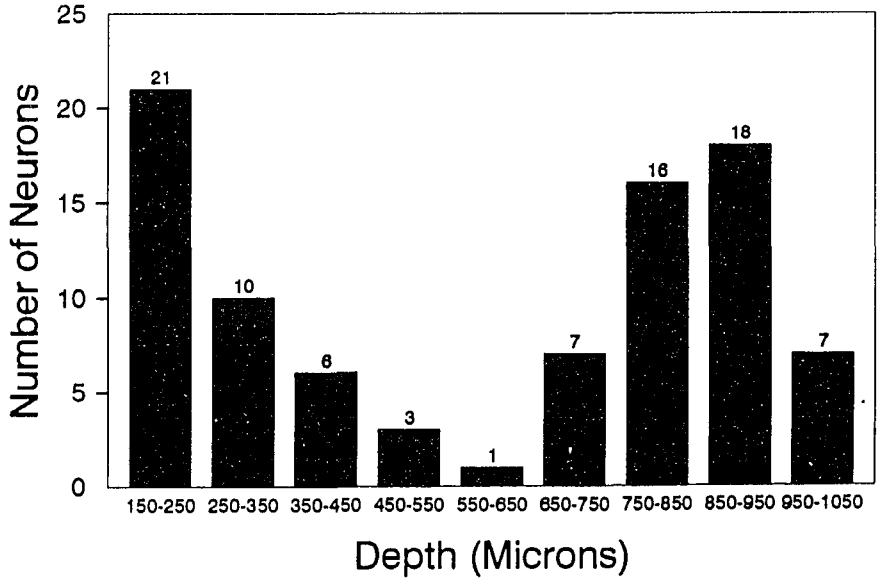
Responses of single olfactory bulb neurons to amino acids were highly reproducible over time (up to 5 hours). Responses to any

Fig. III.1. (*A*) Photomicrograph of a 50 μm coronal section of the olfactory bulb of the channel catfish showing mitral cells labelled by diI. Dorsal is upward. (*B*) Distribution of recording sites within the olfactory bulb measured from the calibrated hydraulic microdrive.

A

100 uM

B


Number of Neurons
25
20
15
10
5
0
21
10
6
3
1
7
16
18
7
150-250
250-350
350-450
450-550
550-650
650-750
750-850
850-950
950-1050
Depth (Microns)

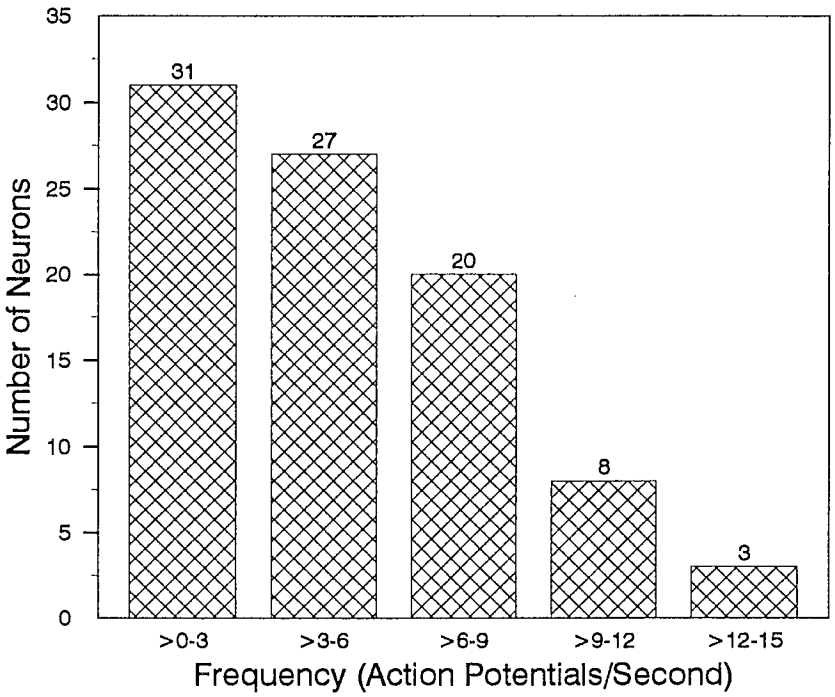


Fig. III.2. Frequency distribution of the spontaneous activity of 89 recorded cells.

Fig. III.3. Electro-olfactogram (EOG) recorded from the channel catfish olfactory epithelium (upper traces; negative downward) and action potentials recorded simultaneously from a single cell (*neuron* #19) in the olfactory bulb (lower traces). (*A*) Null response to control water; (*B*) Excitatory response to 10^{-4} M Met; (*C*) Suppressive response to 10^{-3} M Glu; (*D*) Null response to 10^{-4} M Lys. The initial positive deflection (asterisk) of the EOG record that precedes the onset of the EOG response is an experimental artifact due to the slight pressure pulse created by switching the stimulus injection valve. The onset of the EOG response (arrowhead) was used to indicated stimulation onset. The neural activity was divided into 3 periods. Period I was the prestimulation (spontaneous activity) period; period II was the stimulus delivery period; and period III was the stimulation period, judged from the EOG waveform. In this and subsequent figures, the prestimulation and stimulus delivery periods were separated by dashed lines, and the stimulus delivery and stimulation periods were separated by dotted lines.

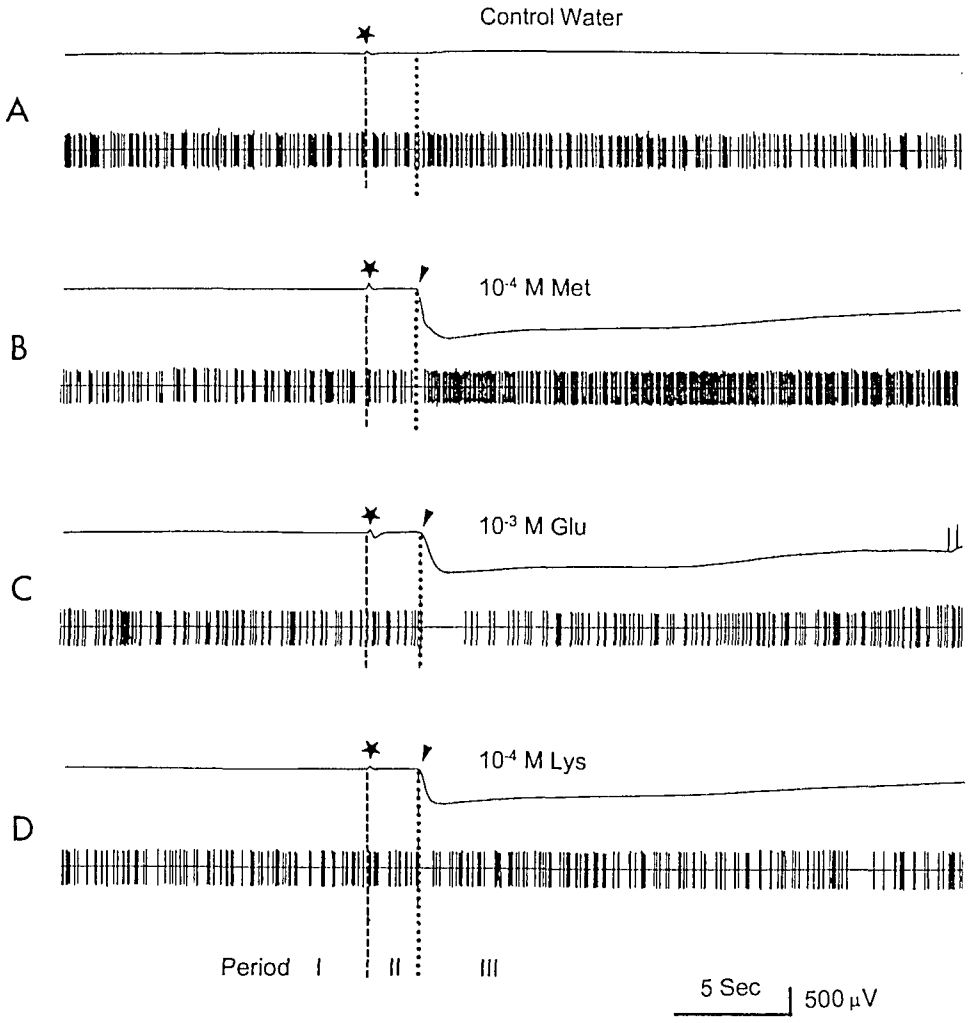
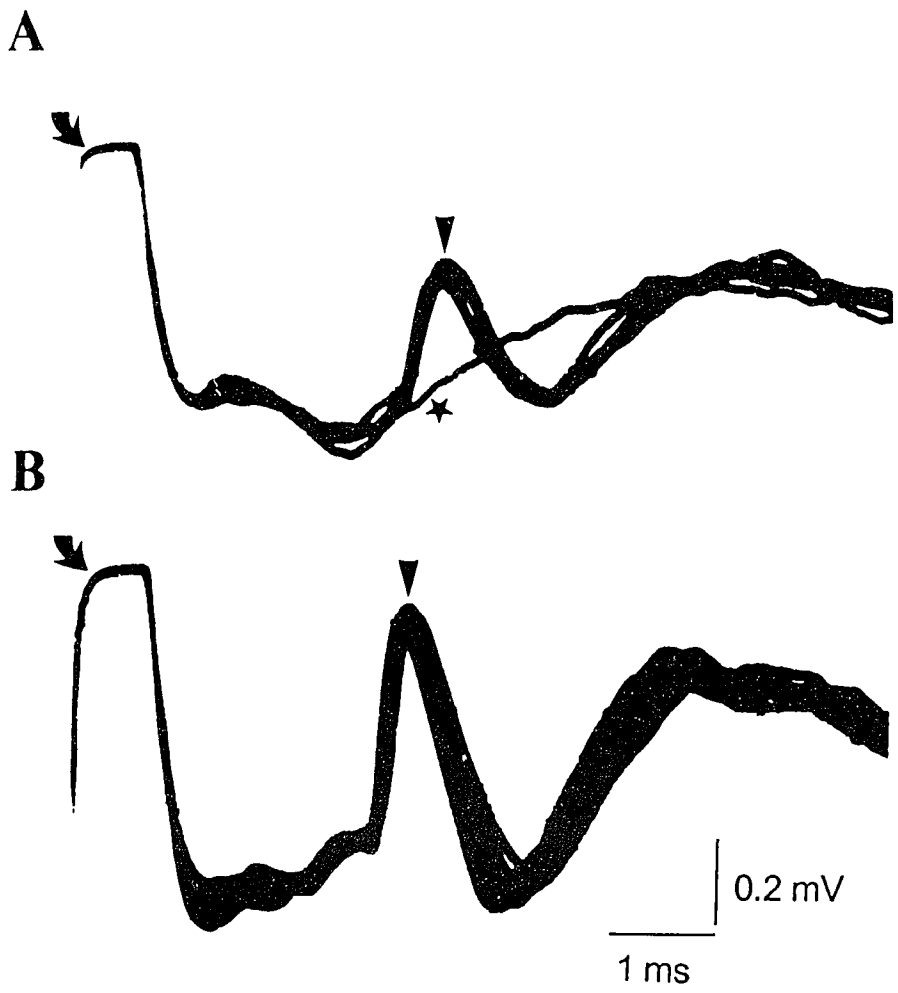

Control Water
A
B
10^{-4} M Met
C
10^{-3} M Glu
D
10^{-4} M Lys
Period I II III
5 Sec
500 μV

Fig. III.4. Responses to electrical stimulation of the olfactory tract. (A) Responses (6 overlapping traces) of cell A to 0.5 mA, 700 μs stimulation of the olfactory tract. Cells in the subset of electrical stimulation experiments were identified separately from those studied in chemical stimulations. Star indicates a response to an electrical stimulus that failed to evoke an action potential. (B) Responses (11 overlapping traces) of cell B to 0.5 mA, 700 μs stimulation of the olfactory tract. Arrows indicate stimulus artifacts, and arrowheads indicate evoked action potentials.


A
B
0.2 mV
1 ms

stimulus never changed from excitation (E) to suppression (S), or *vice versa* over time (Fig. III.5; Table III.1). In only three out of 14 cases in which a given amino acid was tested at least four times did the response of an olfactory bulb neuron to the same stimulus change from an E or S type to an N type over the analyzed time periods (Table III.1). Examination of temporal patterns of these 14 sets of responses using maximum and minimum inter-spike intervals during 5-s stimulation periods also indicated that the response patterns of the recorded cells to a stimulus over time were highly reproducible (Table III.1).

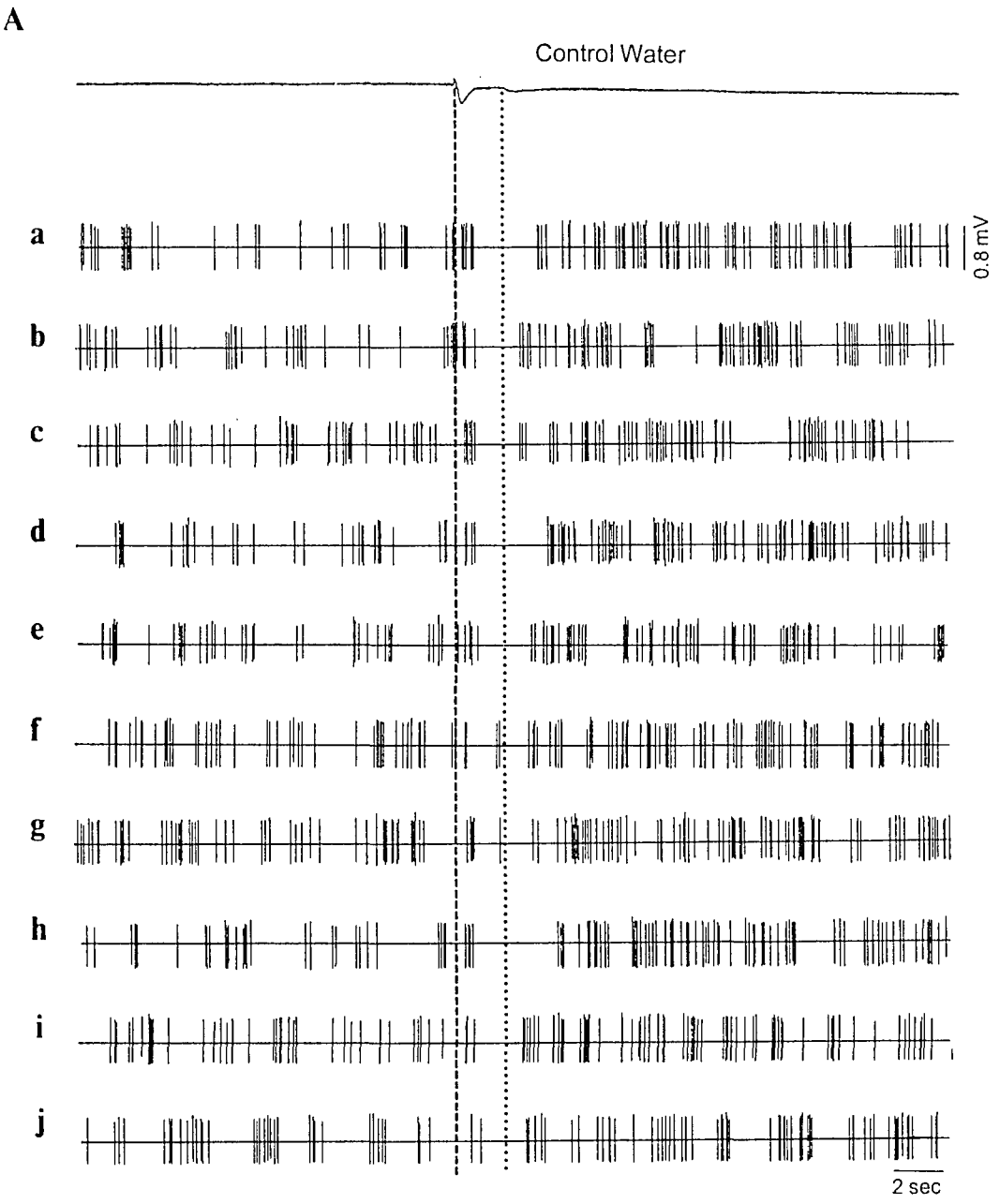
To further illustrate the response reproducibility of the recorded neural activity, the Spearman Correlation, which is sensitive to the temporal pattern of the responses, was used as a descriptive statistic. The average value (Avr_S) of the correlation coefficient (r_S) for the responses of olfactory bulb neurons to 148 pairs of repeated stimuli was 0.43 $\pm$ 0.02 (mean $\pm$ SE; Table III.2). In contrast, the Avr_S value for the responses to 148 pairs of different randomly selected stimuli was 0.06 $\pm$ 0.03 (mean $\pm$ SE).

Examinations of the recordings showed that there were no consistent latencies for the appearance of the initial stimulus-driven action potentials from single olfactory bulb neurons to repeated trials of the same stimulus (see samples in Fig. III.5).

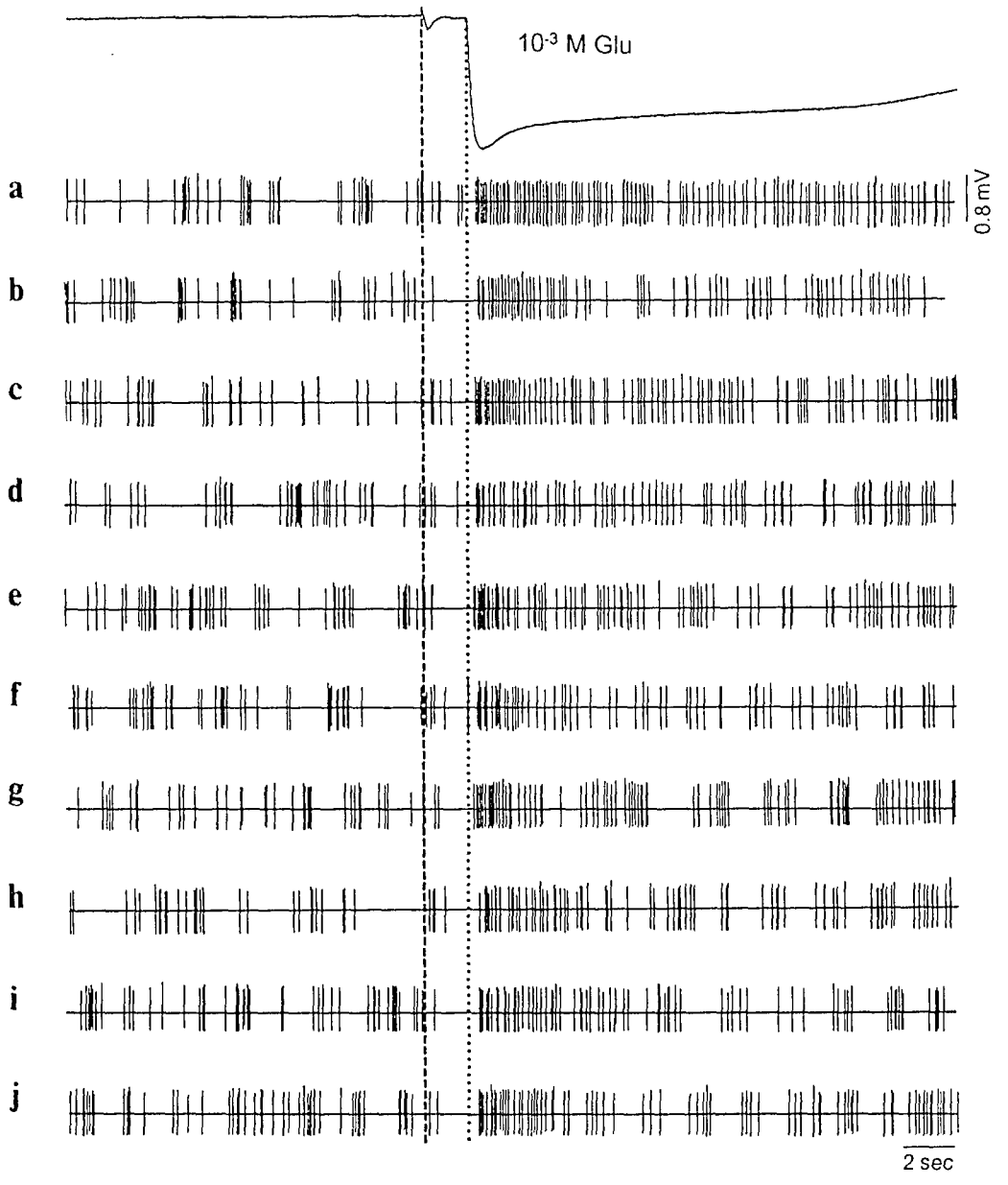
Different Response Types

Based on the interrupted time-series analysis, different types of responses were elicited (1) from the same neuron by different amino acids (Fig. III.6) and (2) from different neurons by the same amino

Fig. III.5. Response reproducibility. (A) Although the switching of the manual sample injection valve elicited some changes in the spontaneous activity, the neuron (*neuron* #88) showed no significant responses (null responses, N) to water controls in all 10 repeated trials (a-j). (B) Constant excitatory (E) responses were evoked from the same neuron as in *A* by 10^{-3} M Glu solutions in all 10 repeated trials (a-j). (C) Suppressive (S) responses were consistently elicited from another neuron (*neuron* #89) by 10^{-4} M Met solutions in all 6 repeated trials (a-f). See figure III.3 for details concerning the prestimulation, stimulus delivery, and stimulation periods. For each series of electrophysiological recordings (A-C), only one EOG trace is shown, which was obtained from the first trial of each series.


A
Control Water
a
b
c
d
e
f
g
h
i
j
0.8 mV
2 sec

B



C

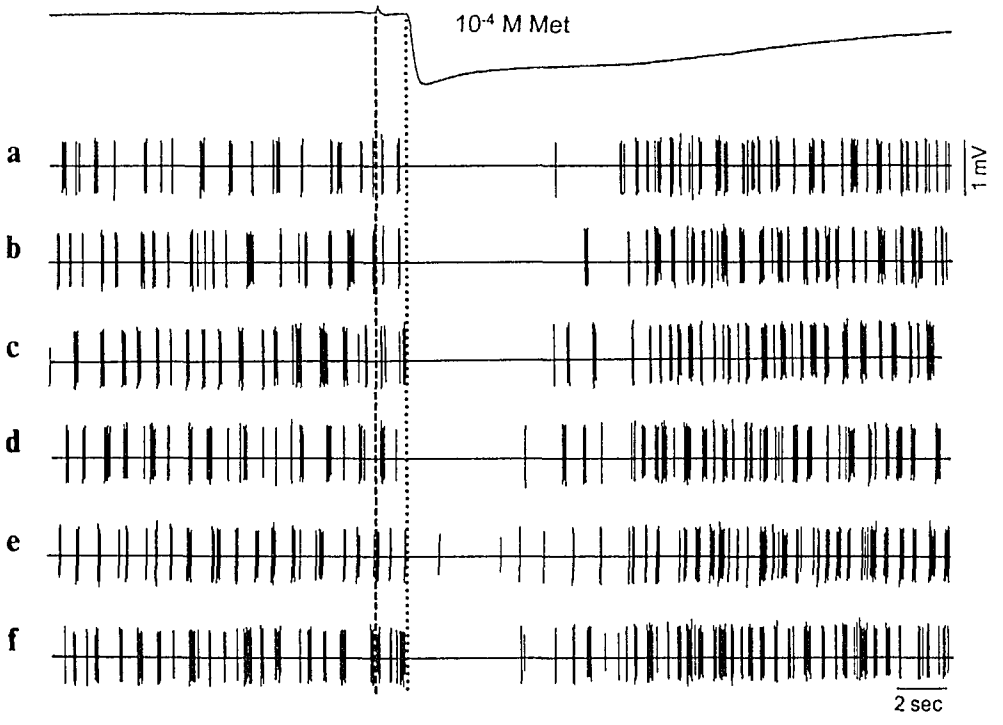


Table III.1. *Response Reproducibility*

Neuron #	AA*	Trial Number 1	2	3	4	5	6	Control Water
1	Lys	S(4)**	S(16)	S(32)	N(48)	-	-	N
		216	126	29	39			30
		842	470	597	407			255
12	Met	E(4)	E(28)	E(112)	E(116)	-	-	N
		14	20	25	33			24
		159	199	233	214			677
25	Met	N(4)	N(36)	N(68)	N(96)	-	-	N
		36	24	18	24			16
		537	278	549	828			537
25	Glu	N(52)	S(72)	S(188)	S(192)	-	-	N
		19	30	20	35			16
		406	660	836	602			537
37	Glu	S(20)	S(32)	S(68)	S(96)	S(132)	S(316)	N
		na	na	na	na	na	na	27
		5000	5000	4910	4975	5000	4885	1339
37	Met	E(80)	E(156)	E(180)	E(196)	-	-	N
		24	23	22	21			27
		461	882	556	411			1339
37	Arg	S(48)	S(184)	S(216)	S(272)	-	-	N
		2107	na	na	na			27
		2893	5000	5000	4971			1339
37	Ala	E(4)	N(36)	E(200)	E(212)	-	-	N
		20	32	23	20			27
		1125	564	594	2000			1339
38	Asp	S(44)	S(76)	S(96)	S(148)	-	-	N
		na	na	na	225			105
		4848	5000	5000	1926			636
42	Ala	E(4)	N(28)	E(148)	E(180)	-	-	N
		8	13	7	7			15
		393	588	448	77			612
42	Met	S(68)	S(92)	S(112)	S(160)	-	-	N
		13	65	12	12			15
		1624	1055	1280	1312			612
87	Glu	S(4)	S(8)	S(12)	S(16)	S(20)	S(28)	-
		na	81	80	81	250	92	
		5000	308	4459	4450	4177	4313	
88	Glu	E(72)	E(80)	E(84)	E(92)	E(96)	E(104)	N
		17	33	41	12	13	32	39
		293	400	502	716	350	531	1461
89	Met	S(4)	S(8)	S(12)	S(16)	S(20)	S(24)	-
		na	na	na	31	750	16	
		5000	5000	5000	4704	2560	4484	

* AA, amino acid stimuli.

** E, S, and N are response classifications representing excitation, suppression, and null responses, respectively. Numbers in parentheses to the right indicate the time in minutes from the initial response of that unit to a stimulus. Numbers below the response classifications are the minimum and maximum inter-spike intervals in ms during the five second response period. "na", not applicable. "-", no tests conducted. Only those amino acids which were tested at least four times were listed in the table.

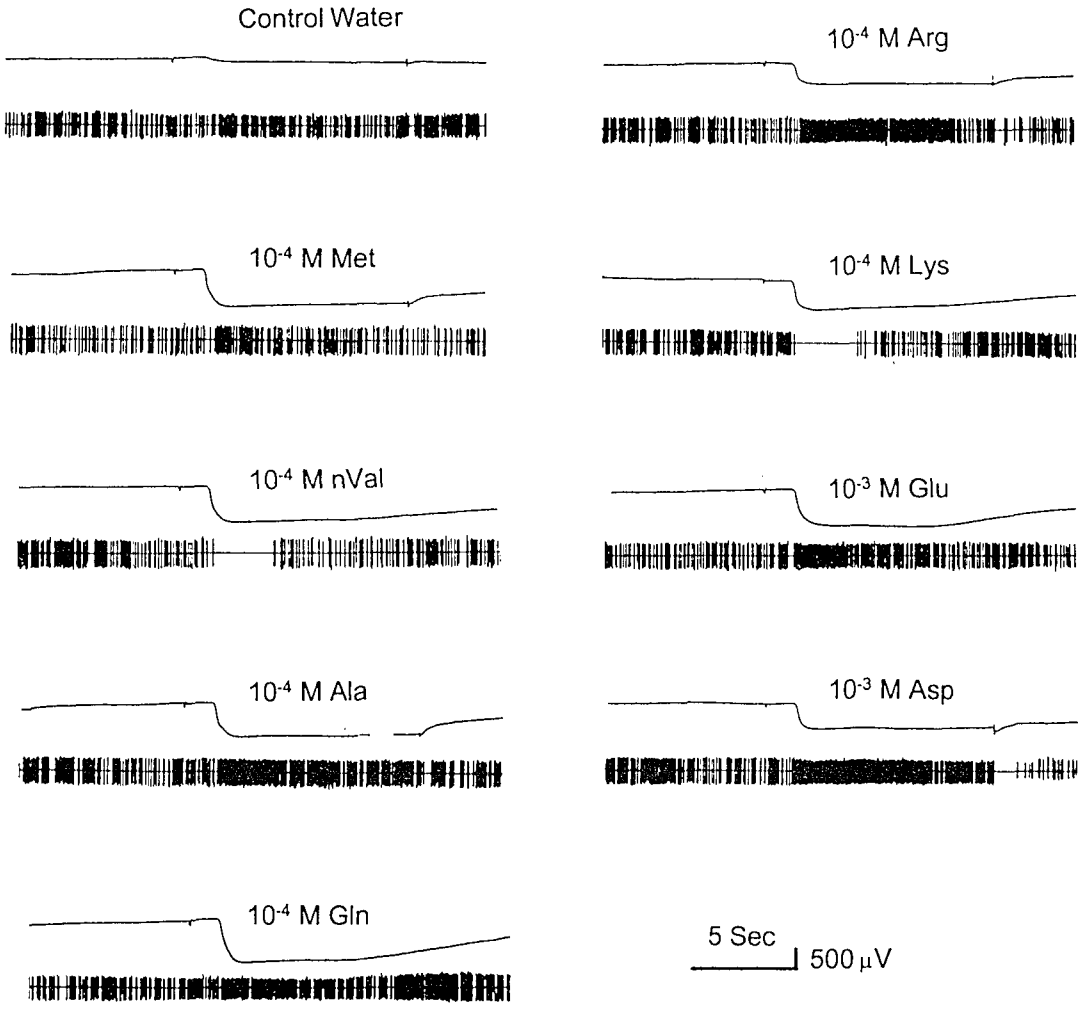
Table III.2. *Response Consistency and Discrimination (Spearman Correlation Analysis)*

Groups	n*	Avr_S**	S.E.
A: Consistency (Same Stimuli)	148	0.43	0.02
B: Discrimination (Different Stimuli)	148	0.06	0.03

* Number of stimulus pairs which evoked action potentials that were subjected to the Spearman correlation test. See Methods and Materials for details.

** Avr_S, average r_S, the Spearman correlation coefficient. Notice that the critical value for N=30 (N, the number of bins) is 0.305 (α=0.05, one-tailed). Thus, if an obtained r_S value was greater than the critical value, the responses to the stimulus pair were determined to be similar to each other.

Fig. III.6. Representative recordings showing EOG and neural responses to a water control and to eight amino acids (*neuron* #47). The neuron responded to water with a null response; to Met, Gln, Ala, Arg, Glu and Asp with excitatory responses; and to nVal and Lys with suppressive responses.


Control Water
10-4 M Arg
10-4 M Met
10-4 M Lys
10-4 M nVal
10-3 M Glu
10-4 M Ala
10-3 M Asp
10-4 M Gln
5 Sec
500 µV

acid. Of the 337 stimulus applications, excluding repeated (multiple) presentations and intensity-response experiments, 28% of the responses were classified as excitatory, 33% as suppressive and 39% as null. For 41 olfactory bulb neurons that were tested with 5 to 8 different amino acids, only 2 neurons (number #13 and #41) responded with the same response type (suppression) to the tested amino acids. The 39 other cells showed different response types to the different amino acids tested (Fig. III.7A).

Each amino acid tested elicited all three types of neural activities (i.e., E, S and N) from different olfactory bulb neurons (Table III.3; Fig. III.7B). Chi-square analysis conducted on the number of neurons grouped according to response types and to the amino acids administrated indicated that there was no significant relationship (X=12.38, P>0.05, df=14) between any particular response type and any specific amino acid.

MANOVA tests were conducted to examine the overall relationship between the responses to pairs of amino acids to determine whether single olfactory bulb neurons could be classified into different response groups according to the overall relation between responses and stimuli. With the single exception of responses to Met and nVal, the overall responses of 65 cells to pairs of amino acids were not significantly correlated (P > 0.05, Table III.4).

Intensity-Response Functions

A total of 37 intensity-response functions of 28 olfactory bulb neurons were tested with stimuli covering at least a thousand-fold change in concentration (Table III.5). Response types, judged by the

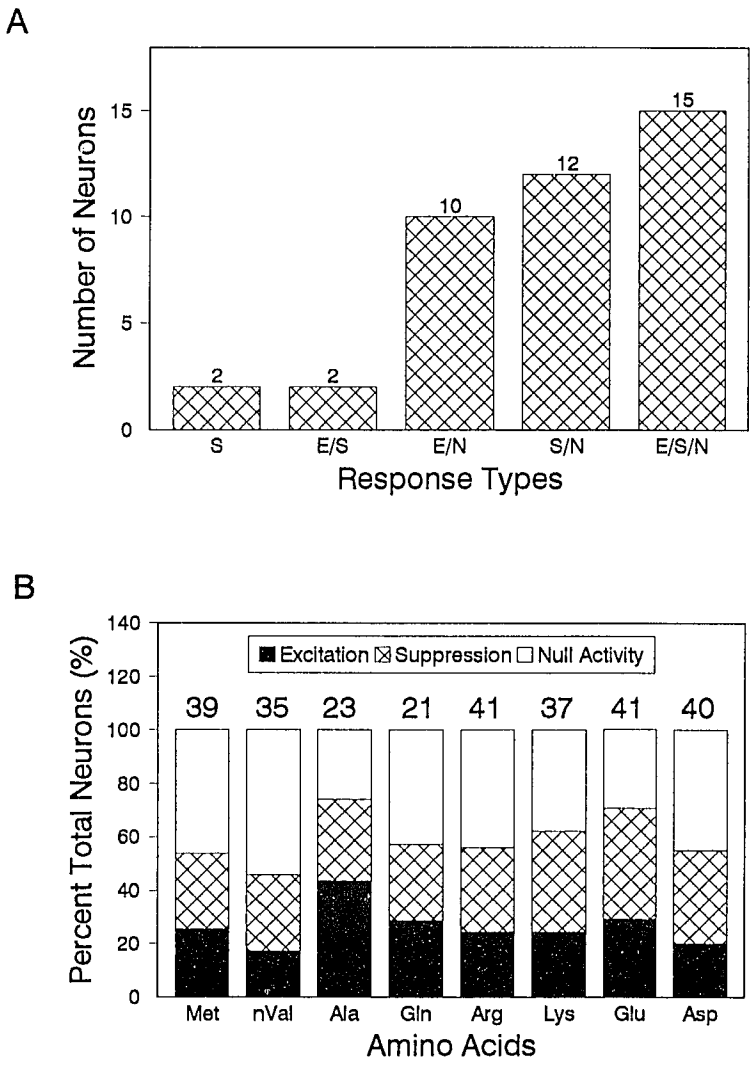


Fig. III.7. Distribution of response types. Only neurons tested with ≥ five amino acids were included in this figure. (*A*) Distribution of response types to different amino acids. E/S: responded to different amino acids with either excitation or suppression; E/N: responded to different amino acids with either excitation or null; S/N: responded to different amino acids with either suppression or null; E/S/N: responded to different amino acids with either excitation, suppression or null. (*B*) Distribution of N, E, and S response types to each amino acid stimulus tested across the sampled neurons. Numbers above each bar are the total number of cells tested with the corresponding stimulus.

Table III.3. *Response Types to Different Amino Acids (Interrupted Time-Series Analysis)*

Neuron	Met	nVal	Ala	Gln	Arg	Lys	Glu	Asp
1	N*	N	-	-	N	N	E	E
7	E	N	-	-	N	E	S	N
8	N	S	-	-	E	N	N	N
11	N	S	-	-	E	N	E	N
12	E	E	-	-	S	N	N	E
13	S	S	-	-	S	S	S	S
14	N	N	-	-	E	N	N	N
15	N	S	-	-	E	E	E	S
16	S	S	-	-	N	S	S	S
17	S	S	-	-	S	S	S	E
18	S	S	-	-	N	N	S	E
19	E	E	-	-	N	N	S	N
20	S	N	-	-	N	E	E	N
21	N	N	-	-	N	S	E	N
22	N	E	-	-	N	E	N	S
24	N	N	N	N	N	N	S	N
25	N	N	N	N	N	E	E	E
26	N	S	-	-	S	S	N	S
27	N	N	-	-	N	N	N	S
28	N	N	-	-	E	E	N	E
30	N	N	N	N	S	S	S	S
33	S	N	N	S	N	S	S	N
37	E	N	E	N	S	S	S	N
38	N	E	E	E	E	E	S	S
41	-	-	S	S	S	S	S	S
42	S	N	E	S	N	S	S	S
44	S	N	S	N	S	N	N	S
47	E	S	E	E	E	S	E	E
48	E	-	E	N	N	-	N	-
49	N	E	E	E	E	N	E	N
51	-	-	E	N	N	-	E	N
53	E	-	S	-	S	-	N	S
54	N	N	S	N	N	N	N	N
55	S	S	S	S	S	S	S	N
56	S	-	N	S	S	S	S	S
57	E	N	E	E	N	E	E	N
58	E	N	E	E	E	E	E	E
61	N	-	S	-	S	-	S	S
63	E	N	S	S	S	S	S	N
64	S	N	N	N	N	N	N	N
65	N	E	E	E	E	N	E	N

* E, S, and N indicate excitatory, suppressive, and null responses, respectively. - indicates no tests conducted.

Table III.4. *Relationship between Overall Responses to Stimulus Pairs Determined by MANOVA*[*]

AA	Met	nVal	Ala	Gln	Arg	Lys	Glu	Asp
Met		.0218[**]	.6898	.7559	.7180	.6640	.8666	.1309
nVal	43		.2332	.1210	.2992	.0922	.2137	.1034
Ala	22	18		.3536	.5105	.5068	.4724	.6006
Gln	18	17	24		.1477	.3110	.5833	.9406
Arg	47	36	25	20		.6816	.5418	.4290
Lys	39	36	20	18	41		.7221	.7907
Glu	40	33	26	21	41	36		.5296
Asp	37	32	22	19	41	35	39	

[*] MANOVA were conducted on responses to all pairs of L-amino acids. Numbers below the diagonal indicate the neuron numbers tested, and above the diagonal are *P* values.

[**] With the exception of the responses to Met and nVal, MANOVA tests indicate that overall responses to all pairs of amino acids were significantly different.

Table III.5. *Response Types to Different Concentrations of Stimuli (Interrupted Time-Series Analysis)*

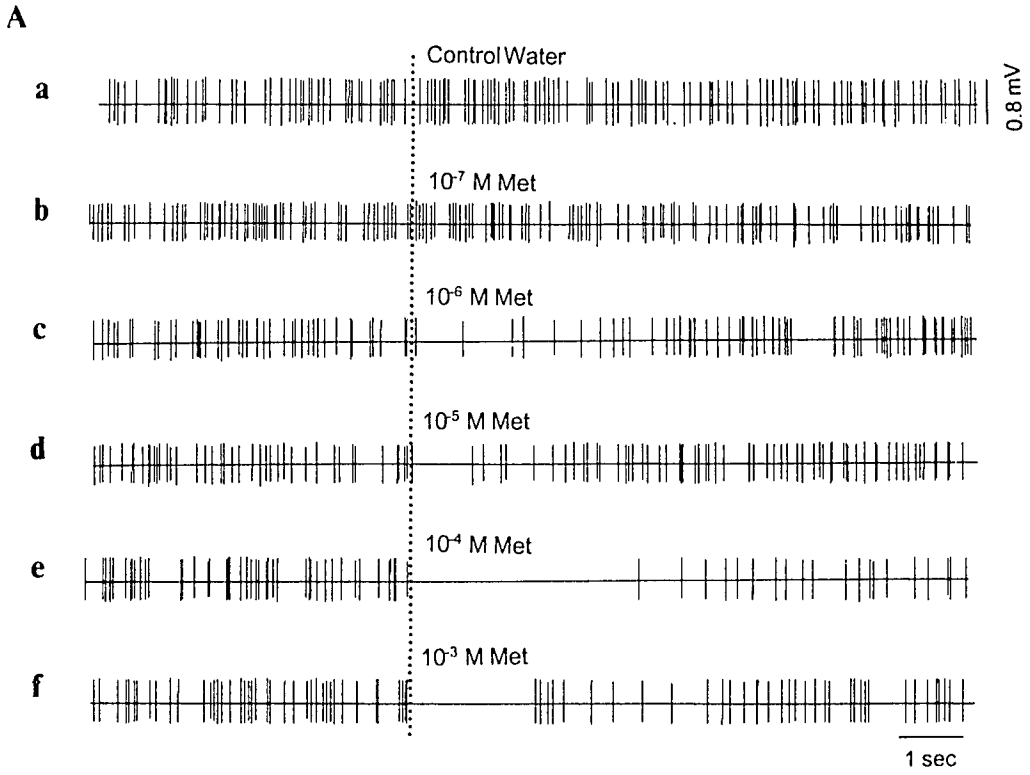
Neuron	Stimulus	Log Molar Concentration					Patterns
		-7	-6	-5	-4	-3	Assigned
25	Arg	-*	N(2)	N(2)	E(2)	E(2)	E1
42	Ala	-	N(1)	N(2)	E(2)	E(1)	E1
71	nVal	N(2)	N(2)	N(2)	E(2)	E(1)	E1
76	Met	-	-	N(1)	N(1)	E(1)	E1
12	Met	N(2)	E(2)	E(2)	E(2)	E(2)	E2
37	Met	N(1)	E(2)	E(2)	E(2)	N(1)	E2
57	Met	E(2)	E(2)	E(2)	E(1)	-	E2
67	Arg	N(1)	N(1)	E(1)	E(1)	E(1)	E2
69	Met	E(2)	E(2)	E(2)	N(2)	N(1)	E2
70	Gln	N(2)	N(2)	E(2)	N(3)	N(2)	E2
81	Lys	N(2)	N(2)	E(2)	N(4)	N(1)	E2
68	Lys	E(2)	N(2)	N(2)	N(2)	E(2)	E3
73	Met	E(1)	E(1)	E(1)	E(1)	E(1)	E3
79	Arg	E(2)	N(2)	N(2)	N(1)	N(1)	E3
84	Ala	E(2)	E(2)	E(2)	E(1)	N(2)	E3
86	Arg	E(2)	E(2)	E(2)	N(2)	N(2)	E3
25	Glu	-	N(2)	N(2)	N(2)	S(3)	S1
37	Arg	N(2)	S(2)	S(2)	S(2)	S(2)	S1
72	Met	N(1)	N(1)	N(1)	S(2)	S(2)	S1
74	Lys	-	-	S(1)	S(1)	S(1)	S1
77	Glu	-	-	N(1)	N(1)	S(1)	S1
80	Lys	N(2)	S(2)	S(1)	S(1)	S(1)	S1
80	nVal	S(1)	S(1)	S(2)	S(2)	S(2)	S1
82	Met	N(2)	S(2)	S(2)	S(2)	S(2)	S1
85	Met	N(1)	N(1)	S(1)	S(1)	S(1)	S1
86	Ala	S(1)	S(1)	S(1)	S(1)	S(1)	S1
86	Met	N(2)	S(2)	S(2)	S(2)	S(2)	S1
86	nVal	N(2)	S(2)	S(3)	S(2)	S(3)	S1
37	Glu	-	N(1)	S(2)	S(3)	S(3)	S2
38	Asp	-	N(2)	N(2)	S(2)	S(3)	S2
76	Arg	N(2)	N(2)	S(2)	N(2)	N(2)	S2
78	Asp	N(1)	N(1)	S(2)	N(2)	N(2)	S2
29	Met	N(1)	N(1)	-	N(1)	-	N
66	Arg	N(1)	N(1)	N(1)	-	-	N
75	Lys	N(1)	N(2)	N(2)	N(2)	N(2)	N
81	Glu	N(1)	N(1)	N(1)	N(1)	N(1)	N
83	Ala	N(2)	N(2)	N(2)	N(2)	N(1)	N

* Numbers in parentheses indicate the number of tests. "-" indicates no tests conducted. The patterns for intensity-response functions were assigned according to the relationship between stimulus concentration and the evoked response magnitude as shown in Fig. III.8; e.g., the intensity-response functions for E1 were shown in Fig. III.8A1. See text for details.

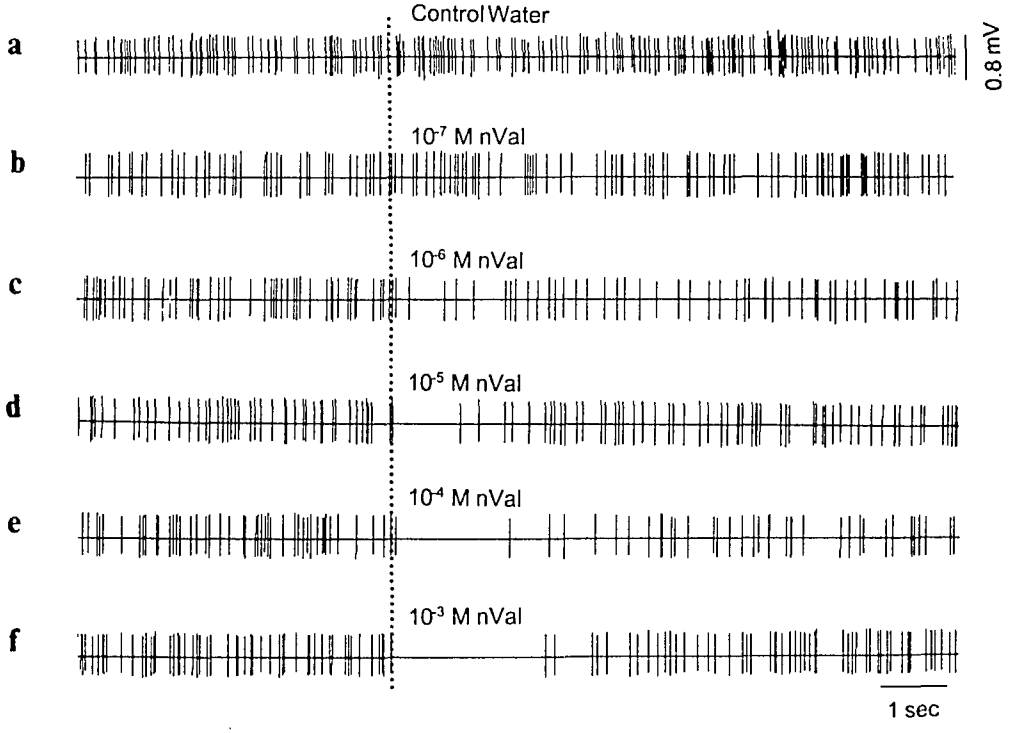
interrupted time-series analysis, were compared among different concentrations of a given stimulus. Estimated threshold concentrations, indicated by the first detectable stimulus-driven response in intensity-response functions, ranged from 10^{-7} to 10^{-3} M and were different from neuron to neuron for a particular stimulus and from stimulus to stimulus for a particular neuron (Table III.5; Fig. III.8). With increasing concentrations from 10^{-7} to 10^{-3} M, response types never changed from excitation (E) to suppression (S), or *vice versa*, for a given stimulus. However, there were some cases (n=10 out of 37) that the response types changed from E or S to N. Different relationships between stimulus concentration and response occurred for different neurons and stimuli. For some intensity-response functions, the response types did not change with increasing concentrations of a suprathreshold stimulus. For a few cells (3 of 37), the intensity-response functions were non-linear (i.e., with increasing concentration, the neural activities for these neurons changed from N to E and then back to N) (Table III.5).

The intensity-response functions were further examined by plotting the concentration vs. the evoked standardized response magnitude (Fig. III.9). For excitatory responses, three patterns of intensity-response functions were observed: (a) E1: the response magnitude increased with increasing stimulus concentrations (Fig. III.9Aa); (b) E2: the response magnitude increased and then decreased with increasing stimulus concentrations (Fig. III.9Ab); and (c) E3: the response magnitudes decreased with increasing stimulus concentrations (Fig. III.9Ac). For suppressive responses, two

Fig. III.8. Responses of a single cell (*neuron* #86) to increasing log steps of amino acid concentrations. The dotted lines indicate the start of the response as indicated by the onset of the simultaneously recorded EOG (not shown). (A) The cell did not respond to a water control (a) or to 10^{-7} M Met (b), but was suppressed by 10^{-6} to 10^{-3} M Met (c-f). (B) The same cell did not respond to a water control (a) or to 10^{-7} M nVal (b), but was suppressed by 10^{-6} to 10^{-3} M nVal (c-f). (C) The same cell did not respond to a water control (a), but was excited by 10^{-7} to 10^{-5} M Arg (b-d); however, did not respond to 10^{-4} and 10^{-3} M Arg (e-f).


A
a
Control Water
0.8 mV
b
10-7 M Met
c
10-6 M Met
d
10-5 M Met
e
10-4 M Met
f
10-3 M Met
1 sec

B


Control Water
0.8 mV
a
10^{-7} M nVal
b
10^{-6} M nVal
c
10^{-5} M nVal
d
10^{-4} M nVal
e
10^{-3} M nVal
f
1 sec

C

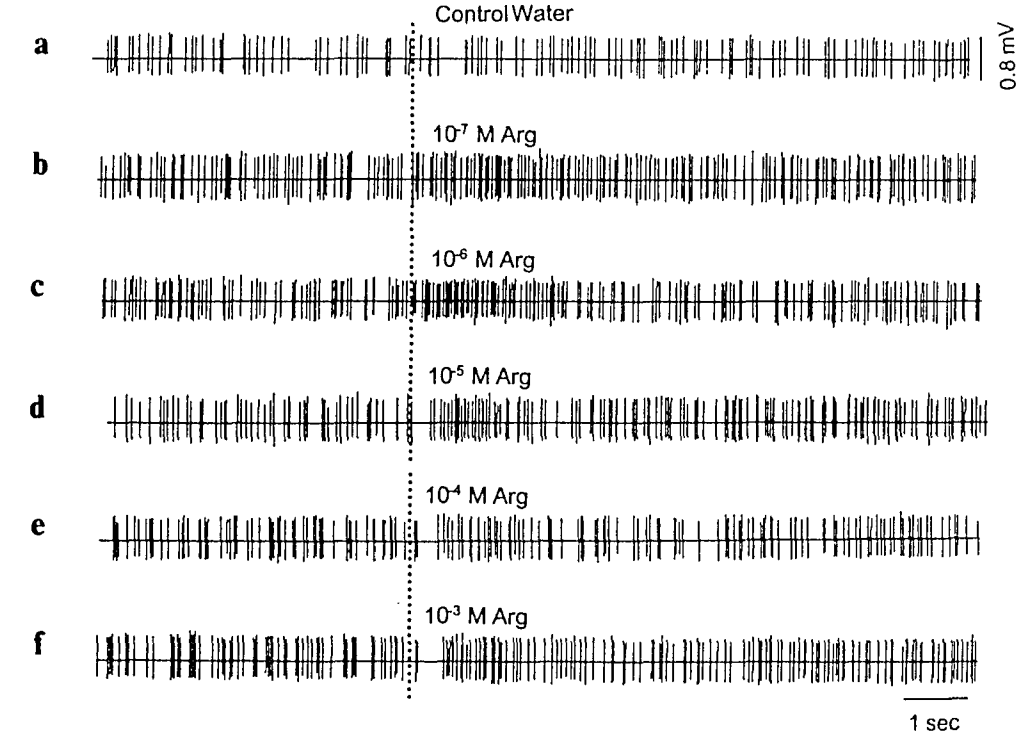
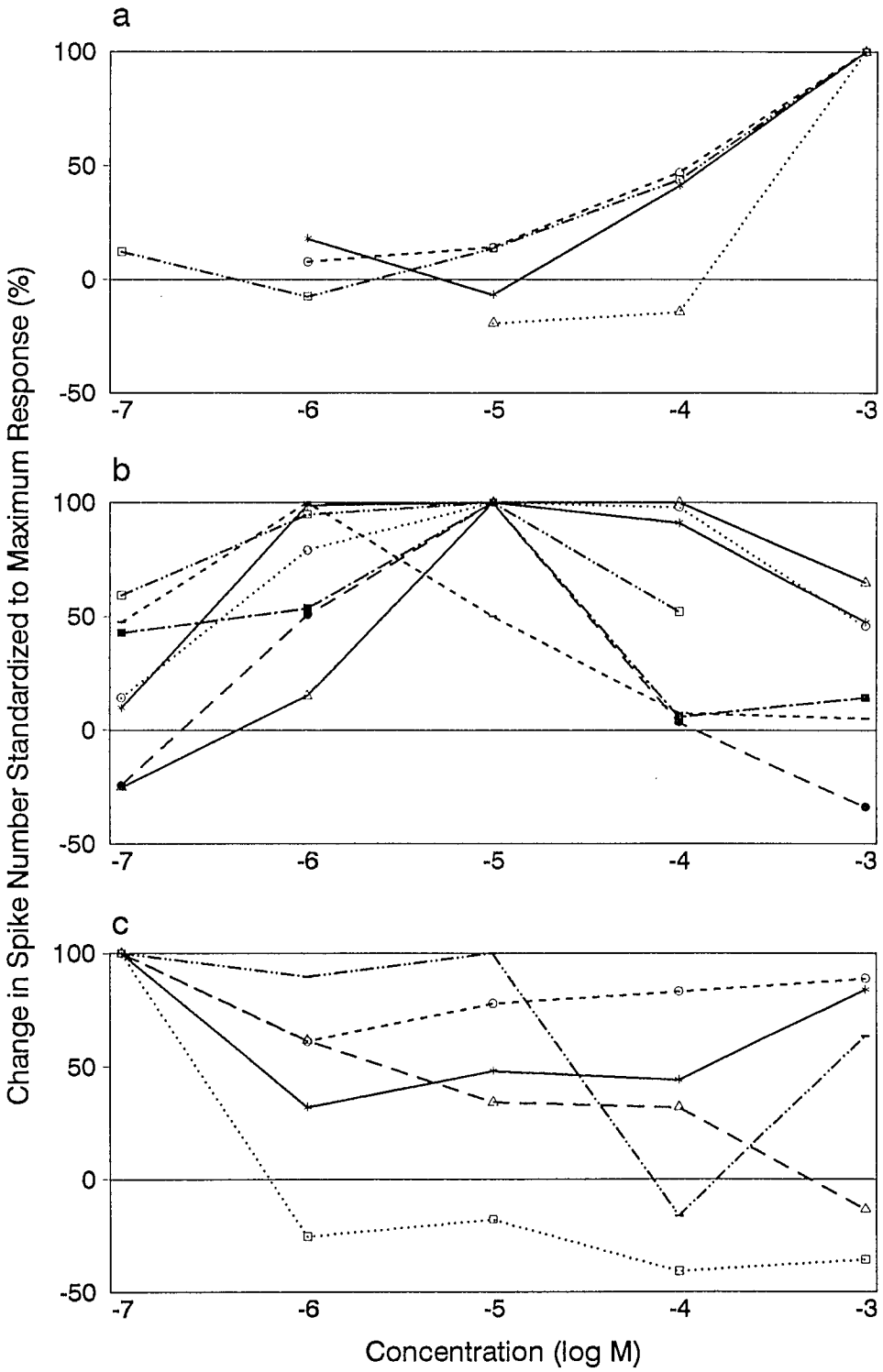
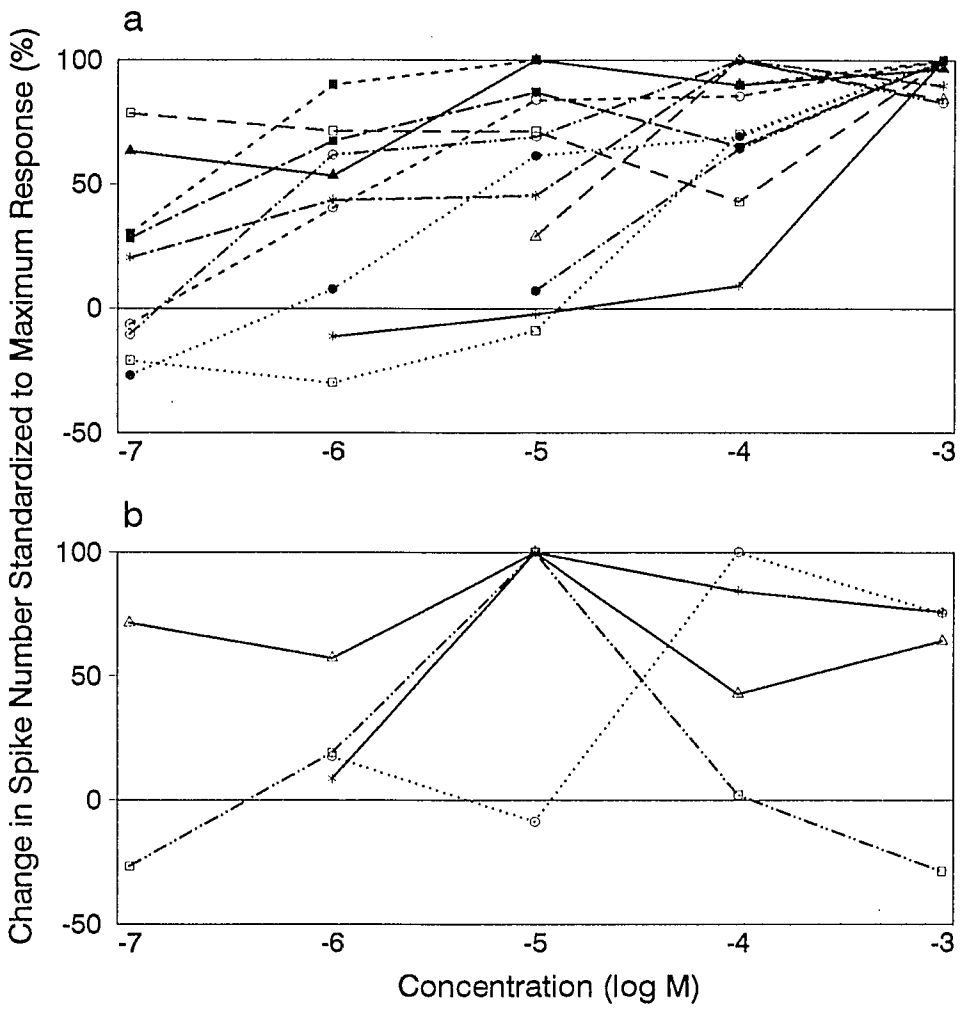

Control Water
0.8 mV
a
10-7 M Arg
b
10-6 M Arg
c
10-5 M Arg
d
10-4 M Arg
e
10-3 M Arg
f
1 sec

Fig. III.9. Standardized Intensity-response functions. (*A*) Excitatory responses to amino acids at different concentrations. (*a*) The E1 intensity-response pattern. (*b*) The E2 intensity-response pattern. (*c*) The E3 intensity-response pattern. (*B*) Suppressive responses to amino acids at different concentrations. (*a*) The S1 intensity-response pattern. (*b*) The S2 intensity-response pattern. Response magnitudes of cells to amino acid stimuli were calculated by subtracting the number of action potentials occurring during a 5-s prestimulation period from that occurring during a 5-s stimulation period. Averaged responses were used for multiple trials. The maximum response to a stimulus was standardized to 100% and the responses to the same stimulus at other concentrations were scaled to the appropriate percentages. The data sets in this figure were the same as those for Table III.5, in which the data were presented as response types only.

A: Excitatory Responses



B: Suppressive Responses



patterns of intensity-response functions were observed with increasing stimulus concentrations: (a) S1: the amount of suppression increased with increasing stimulus concentrations (Fig. III.9Ba); and (b) S2: the amount of suppression increased and then decreased with increasing concentrations (Fig. III.9Bb).

DISCUSSION

Identity of Recorded Olfactory Bulb Neurons

Three pieces of evidence support the assumption that the majority of the recorded neurons in this study were mitral cells. First, the depth distribution of the recording electrodes in the olfactory bulbs (Fig. III.1*B*) were compatible with the locations of the cell bodies of mitral cells observed in the histological sections (Fig. III.1A; unpublished data). Secondly, the large (>350 μV) and relative constant amplitudes of the action potentials suggested that they were elicited from the large mitral cells (Duchamp-Viret et al., 1989; Reinken and Schmidt, 1986; Mair, 1982b). Thirdly, approximately 70% of the units encountered in a subset of experiments were driven with short and constant latencies by electrical stimulation of the olfactory tract, indicating these cells were probably output neurons of the olfactory bulb (Meredith, 1986; Imamura et al., 1992). However, intracellular recording and staining studies must be performed to confirm the cell types suggested in the present study. The remaining 30% bulb units were not necessarily local inter-neurons, but may have been mitral cells that were not driven by the electrical stimulation. Although it is possible that some of the recorded neurons might not have been mitral cells, previous results indicated

that the responses of output neurons of the golden hamster were not different from the remaining neurons of the olfactory bulb (Meredith, 1986).

Spontaneous Activity

All cells studied in the present report were spontaneously active. The average spontaneous frequency of 5.20 ± 3.56 (mean ± SD) action potentials/second was greater than that observed from olfactory bulb neurons of other teleosts [1.4 action potentials/second for *Oncorhychus nerka* (Bodznick, 1978); 1.7 for *Carrassius auratus* (Meredith and Moulton, 1978); 3.6 for *Salmo gairdneri* (Mac Leod, 1976); and 4.2 for *Lota lota* (DØving, 1966)]. The spontaneous frequency of action potentials of olfactory bulb neurons in the channel catfish was considerably less than that indicated for mammals (DØving, 1987; Reinken and Schmidt, 1986; Chaput and Lankheet, 1987; Chaput and Holley, 1979; Katoh et al., 1993), but was greater than that reported for amphibians (Duchamp, 1982; DØving, 1964; Kauer, 1974). Thus, the spontaneous rates of olfactory bulb mitral cells do not reflect an evolutionary relationship among the different species (Duchamp, 1982). Although it was not possible to exclude the possibility that the higher spontaneous frequency of action potentials in the channel catfish compared with other teleosts and amphibians was due to cell damage by the recording electrode, the preparations functioned for 16 to 344 min per cell. The relatively high spontaneous rates of olfactory bulb neurons in the channel catfish have an advantage in olfactory coding since neurons with high spontaneous activity can be modulated (either enhanced or suppressed)

more effectively than those with low spontaneous activity (Duchamp, 1982). Also, the high spontaneous activity of the cells in the olfactory bulb of channel catfish allowed for an easy recognition and classification of suppressive responses.

Criteria for Response

Different criteria have been used in prior studies of single olfactory bulb neurons to determine whether a response occurred. In most previous studies, the response to a stimulus was determined solely by eye, judged through comparing poststimulation periods with prestimulation periods of the recording traces or the reconstructed histograms (Imamura et al., 1992; Katoh et al., 1993; Kauer, 1974; Kauer and Moulton, 1974; Kauer and Shepherd, 1977; DØving, 1987; Mair, 1982b; Mair, 1982a; Mathews, 1972a; Mathews, 1972b). Responses of olfactory bulb neurons, however, may not be obvious upon visual comparison with spontaneous activity. In order to avoid experimenter bias in determining whether a response occurred, specific criteria (Mac Leod, 1976; Meredith, 1986; Bodznick, 1978) and statistical methods (Buonviso and Chaput, 1990; Fischer and Zippel, 1989; Duchamp-Viret et al., 1989; Chaput and Lankheet, 1987; Chaput and Holley, 1985; Meredith and Moulton, 1978; Schild, 1987) were used in other studies. The precision of these statistical methods for determining responsiveness is dependent upon the number of repeated stimulus trials, which was often compromised by the limited recording time/cell. For the present experiments, the interrupted time-series analysis did not require multiple trials and avoided individual bias in determining responsiveness.

Classification of Response Types

Similar to determining whether a response occurred, many of the classification schemes used in previous studies were based on comparing visually the frequency of action potentials occurring during prestimulation and poststimulus periods from either the recorded traces (Imamura et al., 1992; Katoh et al., 1993; Kauer, 1974; Kauer and Moulton, 1974; Kauer and Shepherd, 1977; Duchamp, 1982; Bodznick, 1978; DØving, 1966; Mathews, 1972a; Mathews, 1972b) or from reconstructed activity histograms (Reinken and Schmidt, 1986; DØving, 1987; Meredith, 1986; Meredith and Moulton, 1978; Mair, 1982b; Mair, 1982a). The classification of response types based on visual examination, however, may lead to biased judgments due to individual subjectivity. In more recent studies, statistical tests, such as the median test (Chaput and Lankheet, 1987; Chaput and Holley, 1985), Mann-Whitney U-test (Schild, 1987; Chaput and Lankheet, 1987; Buonviso and Chaput, 1990), and *t* test (Buonviso and Chaput, 1990) were used to classify the response types of neurons. These reports, however, compared only the mean action potential activities of prestimulation and stimulation periods, ignoring the actual time-course of the prestimulation and stimulation periods. The advantage of the interrupted time-series analysis used in present report was that by examining the time-course of both prestimulation and stimulation periods, the time-series statistic not only avoided individual subjectivity, but provided information about the direction (excitation or suppression) of the change from prestimulation activity. However,

a disadvantage of the analysis is that the classification scheme in the present report is incompatible with previous schemes, resulting in difficulties upon comparing the present results with those from previous studies. For example, neural activities of neuron 86 to 10^{-4} and 10^{-3} M Arg (Fig. III.8*Ce,f*) were classified as null responses in the present study, but might have been classified as suppressive responses by only a visual examination of the recorded traces, or classified as weakly excitatory responses by combining visual examinations with measuring the latencies of the first several action potentials (Hamilton and Kauer, 1989).

Compared with previous complex schemes of classifying responses of olfactory bulb neurons (Reinken and Schmidt, 1986; Kauer, 1974; Duchamp, 1982; Meredith, 1986; Meredith and Moulton, 1978), the present classification scheme (i.e., E, S, and N) was simpler, but sufficiently adequate to appropriately describe the responses. Due to the experimental conditions and/or the response characteristics of the species (see discussion in *Quantity Coding*), most of the olfactory bulb neurons studied herein showed uniform excitation, suppression, or null activity to amino acids, and the responses recovered to spontaneous levels following stimulation (For examples see Figs. III.3, III.5 and III.8). Thus, the present scheme of response classification allowed for a consistent evaluation of the recorded action potential discharge to single stimuli and could be used confidently in a subsequent study of responses of olfactory bulb neurons to binary mixtures of amino acids. A similar classification scheme, i.e., classifying responses into excitatory, suppressive, and

null, was utilized in a recent study of rabbit olfactory bulb neurons, although the response types were determined only by visual examinations (Katoh et al., 1993).

It may be unnecessary to use complex classification schemes in categorizing neural responses. For example, in the study of bee antennal lobe neurons, a classification scheme involving four excitatory (E1-E4), four inhibitory (I1-I4) and one null (N) response types was used to classify the responses of the neurons to odors (Sun et al., 1993). However, 92% and 91% of the activities of local and output interneurons, respectively, were classified as either E1, I1 or N responses, which had similar response patterns as the excitatory, suppressive and null response categories utilized in the present study. One factor that may have partially contributed to the use of complex schemes of response classification of olfactory bulb neurons in some previous studies was that the neural activity subsequent to the termination of the stimulus was included in the response. In the present study, stimulation periods (5 seconds), during which the numbers of action potentials occurring was subjected to the interrupted time-series analyses, were within the period of stimulus presentation which lasted approximately 10-15 seconds.

Response Reproducibility

That the responses of olfactory bulb neurons in the channel catfish were reproducible over time (Fig. III.5) is in agreement with previous reports of olfactory bulbar activity (Katoh et al., 1993; Reinken and Schmidt, 1986; Chaput and Holley, 1985; Duchamp, 1982; Meredith and Moulton, 1978; Bodznick, 1978; Kauer, 1974; Kauer and

Shepherd, 1977; Mair, 1982b). In contrast, 27.5% of the responses of goldfish olfactory bulb neurons to repeated stimulations were not reproducible (Schild and Zippel, 1986); however, these results might have been influenced by the short (30 second) interstimulus intervals since the adaptation of both receptors and bulb cells caused by the repetitively presented stimuli could have affected the responsiveness of the cells (Potter and Chorover, 1976; Reinken and Schmidt, 1986; Mair, 1982a). The results of the present study indicated that the 3-min interstimulus intervals allowed for sufficient recovery from adaptation, for stimulus clearance from the olfactory capsule, and thus limited interactions (cross-adaptation) between successive amino acid stimuli.

Excitatory Responses vs. Suppressive Responses

The approximately 1:1 ratio of excitatory to suppressive responses for all stimulus applications in the present report was comparable to that of previous studies (Reinken and Schmidt, 1986; Mac Leod, 1976; DØving, 1987). Excitatory responses, however, were found to exceed suppressive responses by approximately 1.5-2:1 in some other reports (Duchamp, 1982; Meredith and Moulton, 1978; Kauer, 1974). The result of a greater percentage of excitatory than suppressive responses could be the result of the low spontaneous rates (0.5-1.7 action potentials/second) observed in those studies since neurons with low spontaneous rates were less effectively modulated by stimuli for suppressive responses than for excitatory responses (Duchamp, 1982; Sun et al., 1993).

Studies of frog olfactory bulb neurons (specific neuron type not reported) suggested that excitatory responses to stimuli were more important than suppressive responses for olfactory coding (Duchamp-Viret et al., 1989; Duchamp, 1982; Duchamp and Sicard, 1984a; Duchamp and Sicard, 1984b). The present study suggests, however, that suppressive responses may be as important as the excitatory responses in both quality (Fig. III.6; Table III.3) and quantity (Figs. III.8A and III.8B; Table III.5) coding of amino acid information in the channel catfish. Although it was reported that suppressive responses of frog olfactory receptors were rare (Sicard and Holley, 1984; Revial et al., 1982; Revial et al., 1983; Getchell and Shepherd, 1978a; Getchell and Shepherd, 1978b), electrophysiological studies of single olfactory receptor neurons of channel catfish indicated that amino acid stimuli elicited more suppressive responses than excitatory responses (see Chapter II). It is unlikely that the differences reported between fish and amphibians in the relative occurrence of suppressive responses of olfactory receptor and bulbar neurons imply a major difference on the coding of olfactory information between these two classes of vertebrates since hyperpolarizing receptor potentials resulting in a suppression of spontaneous activity were found to be as common as excitatory responses in the mudpuppy (Dionne, 1992). Although species differences may occur, differences in the criteria employed for response classification across different studies is likely a major basis for the differences reported. Due to the low spontaneous rate of frog olfactory bulb neurons (see discussion in

Spontaneous Activity), some suppressive responses might be misjudged as null (Kauer, 1974), and thus the roles of the suppressive responses in olfactory coding might have been underestimated.

Another possibility to explain the discrepancy between previous reports of the lower frequency of reported suppressive responses and those reported here is the classification criteria that defined the response types. For example, some responses classified as suppressive in the present study might have been identified as excitatory responses by other authors. The occurrence of one or two initial action potentials at the onset of the stimulation period followed by an extended suppression of neural activity would be classified as excitatory by Hamilton and Kauer (1989) based on visual examinations with measuring latencies of the initial spikes and post-synaptic potentials associated with the spikes. However, this pattern of neural activity was defined as suppression in the current study since the present classification scheme was designed to measure the overall similarity in the responses patterns to single components and to binary mixtures (Chapter IV).

Two hypothetical mechanisms can account for the observed suppressive responses of mitral cells. Axons of olfactory receptor neurons that were excited by an amino acid stimulus might indirectly suppress the activity of the recorded mitral cell through an inhibitory dendrodendritic synapse from granule cells (Fig. III.10A) (Shepherd, 1979; Katoh et al., 1993). A second mechanism, based on the results of Chapter II, is that recorded mitral cell may be

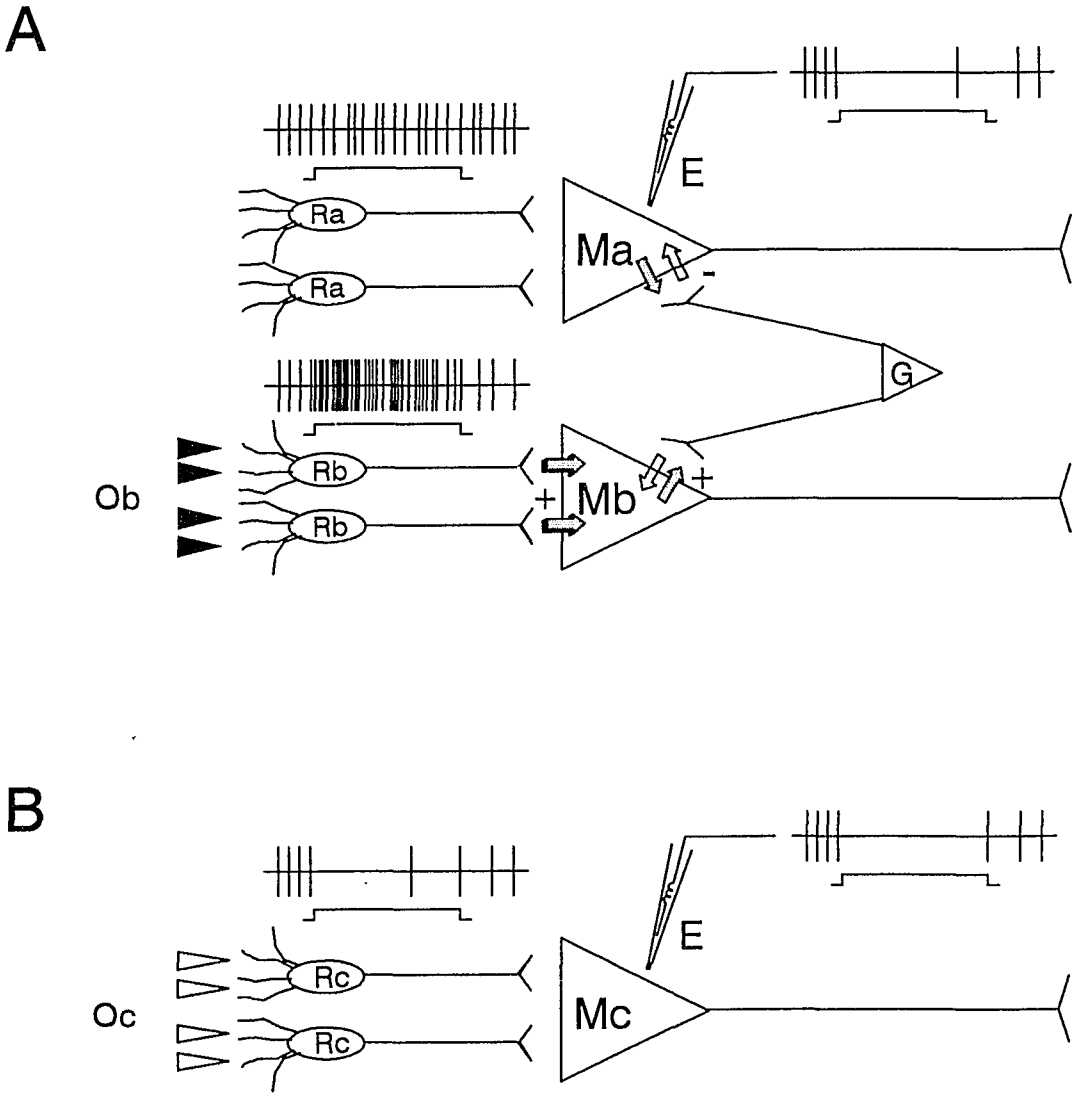


Fig. III.10. Hypothetical schemes to explain the suppressive responses of single olfactory bulb mitral cell (M) of channel catfish. (*A*) Odorant Ob does not affect olfactory receptor neurons, Ra, but excites receptor neurons, Rb, which activate mitral cell, Mb, and granule cell, G, suppress the activity of mitral cell, Ma, whose activity is recorded. (*B*) Odorant Oc directly suppresses olfactory receptor neurons, Rc, which decreases excitatory synaptic input to the recorded mitral cell, Mc, resulting in a suppress of spontaneous activity. E, recording electrode.

suppressed by olfactory receptor neurons which were themselves directly suppressed by the stimulus (Chapter II; Fig. III.10B).

Comparison between Responses of Olfactory Receptor and Bulbar Neurons

Four amino acids were used at the same original concentrations in the present study that were tested in the previous study (Chapter II) of olfactory receptor responses. With a single exception of the percentage of suppressive responses to Ala, the percentages for both excitatory and suppressive responses of olfactory bulb neurons (Figs. III.7*B*) to all four amino acids were greater than those for olfactory receptor neurons (Fig. II.5). Statistical (*t*-test) analysis indicated that the mean percentage of responding olfactory bulb neurons, i.e., combining excitatory and suppressive responses, was significantly greater than that of responding ORNs (df=3; $P<0.005$). The significant increase in the percentage of the responding olfactory bulb neurons compared to ORNs is likely due to the amplifying function of convergence from receptor neurons to bulb neurons. The approximate1000:1 anatomical convergence of olfactory receptor neurons onto mitral cells (Satou, 1990) might be responsible for the higher responsivity of the recorded olfactory bulb neurons. Results from frogs (Duchamp-Viret et al., 1989) and insects (Boeckh and Boeckh, 1979; De Jong, 1988; Boeckh et al., 1984) also supported this assumption that convergence from receptor neurons to mitral cells had an amplifying function in the processing of olfactory signals.

Quantity Coding

RESPONSE TYPES. The present results in the channel catfish that responses of single olfactory bulb neurons did not change from

excitation to suppression, or *vice versa*, across different stimulus concentrations (Fig. III.8; Table III.5) are in agreement with previous studies of responses of olfactory bulb neurons in amphibians (Kauer, 1974; Kauer and Shepherd, 1977; Hamilton and Kauer, 1989; Duchamp-Viret et al., 1989; Duchamp, 1982). Other studies (Meredith, 1986; Meredith and Moulton, 1978; Harrison and Scott, 1986; Wellis et al., 1989; Chaput and Lankheet, 1987; Mair, 1982b), however, indicated that responses of single olfactory bulb mitral cells to a given stimulus could be excitatory or suppressive dependent upon stimulus concentration. With the sole exception of the goldfish study (Meredith and Moulton, 1978), these reports that observed response types changed across stimulus concentrations (Meredith, 1986; Harrison and Scott, 1986; Wellis et al., 1989; Chaput and Lankheet, 1987; Mair, 1982b) tested mammals which were deeply anesthetized to avoid the effects of breathing cycles on the responses. Since anesthetics have profound effects on the response characteristics of neurons within the central nervous system (Reinken and Schmidt, 1986; Spath and Schweickert, 1977; Meredith and Moulton, 1978; Stewart and Scott, 1976; Chaput and Holley, 1979), the change of response types (i.e., excitation to suppression, or *vice versa*) at different concentrations may be partially related to the level of anesthesia. That anesthesia may indeed affect intensity-response functions of olfactory bulb neurons was supported by the findings of Reinken and Schmidt (1986) who showed that olfactory bulb neurons in awake, freely breathing mice responded to a given stimulus at different concentrations with the same response type. Reasons for the different results reported

between goldfish (Meredith and Moulton, 1978) and channel catfish (present study) are unknown, however, differences in the experimental paradigms and the classification of response types between these two studies may partially account for the dissimilarity. For example, the maximum amino acid concentration used in the present report was 10^{-3} M, but goldfish were tested with 10^{-2} M amino acids, a stimulus concentration reported to elicit more suppressive than excitatory responses (Meredith and Moulton, 1978). In the present study, all responses were classified as either excitatory, suppressive or null based on the interrupted time-series analysis, whereas in the goldfish responses were classified into 8 subtypes of excitation and 8 subtypes of suppression based on the form of the reconstructed histograms (Meredith and Moulton, 1978).

INTENSITY-RESPONSE FUNCTIONS. The present results (Fig. III.8) are in agreement with previous reports indicating that the intensity-response functions were different from neuron to neuron and from stimulus to stimulus (Meredith and Moulton, 1978; Meredith, 1986; Duchamp-Viret et al., 1989; Kauer, 1974; Kauer and Shepherd, 1977; Reinken and Schmidt, 1986; Mair, 1982b; Mathews, 1972b). Although the responses of all the cells recorded changed with increasing stimulus concentrations suggesting that the cells might have an ability to discriminate concentration (Figs. III.8 and III.9), it was difficult to summarize quantitatively the relationship between stimulus concentration and magnitude of the evoked response with conventional intensity-response functions. A primary reason for this problem was that the responses of the bulb cells could be either excitatory or

suppressive to the same stimulus across different neurons (Fig. III.9). Thus, most of the previous reports described the intensity-response relationships of only individual neurons (Hamilton and Kauer, 1989; Meredith and Moulton, 1978; Meredith, 1986; Mair, 1982b; Duchamp-Viret et al., 1989; Kauer, 1974; Kauer and Shepherd, 1977; Reinken and Schmidt, 1986; Mathews, 1972b). The present results indicating (1) a large range of estimated threshold concentrations for the same stimulus across different bulb cells (Fig. III.9; Table III.5) and (2) changes in the response magnitude to increases in supra-threshold stimulus concentrations (Figs. III.8 and III.9) provide for an interesting speculation concerning a possible mechanism for coding stimulus intensity. There may exist two systems, "wide-tune" and "fine-tune" systems, for quantity coding in output neurons of the olfactory bulb. The "wide-tune" system codes intensity information simply by the number of output neurons activated; i.e., a stimulus at low concentration activates fewer olfactory bulb output neurons than a stimulus at higher concentrations. The "fine-tune" system, however, codes stimulus intensity information by the response magnitude of the activated neurons; i.e., the activated output neurons responded to stimulus intensity changes with either increased or decreased response magnitudes (Figs. III.8 and III.9). This "fine-tune" system is similar to "concentration tuning" proposed by Kauer (1974), with the exception that Kauer's "concentration tuning" excluded the function of suppressive responses in the coding scheme.

Quality Coding and Response Similarity.

How the olfactory system codes for stimulus quality is a fundamental, but still unresolved issue. A recent report showed that single mitral cells in the dorsomedial region of rabbit olfactory bulbs responded similarly to a group of structurally related fatty acid compounds (Mori et al., 1992). Although the stimuli tested in the present study were all amino acids, previous reports (Bruch and Rulli, 1988; Caprio and Byrd, 1984; Caprio et al., 1989; Kang and Caprio, 1991) clearly indicated the independence of olfactory receptor sites for these compounds. Thus, these stimuli were not treated as a group of functionally similar compounds by the olfactory system. With the single exception in the present report of responses to Met and nVal, the overall responses of the 65 olfactory bulb neurons to all possible pairs of 8 amino acids were not significantly correlated (Table III.4), further confirming that the tested amino acids were not treated as a group of similar compounds by the olfactory system of the channel catfish. The correlation between the responses of olfactory bulb neurons to Met and nVal may, however, be further evidence of the observations by Mori et al (1992) that mitral cells responded similarly to clearly related stimuli. The present results that (a) single olfactory bulb neurons in channel catfish responded to more than one of the tested amino acids (Figs. III.3, III.6, III.7A and III.8; Table III.3), and that (b) most bulb cells sampled had different response spectra (Table III.3) indicated that in the channel catfish it is unlikely that a particular group of olfactory bulb

neurons respond exclusively to a particular compound or a group of similar compounds. This argument is supported by previous studies of single olfactory bulb neurons of various animals in which most of the neurons investigated responded to more than one stimulus odor (Mac Leod, 1976; Meredith, 1981; Meredith and Moulton, 1978; Chaput and Holley, 1985; Mair, 1982b; Wellis et al., 1989; Hamilton and Kauer, 1989; Bodznick, 1978; Duchamp, 1982; Mathews, 1972a; Mathews, 1972b).

Different temporal patterns of responses have been proposed as a candidate code for stimulus quality (DØving, 1966). However, different temporal response patterns could partially result from changes in stimulus concentration (Kauer, 1974; Meredith and Moulton, 1978; Wellis et al., 1989; Mair, 1982b). The present results that different olfactory bulb neurons frequently responded to the same concentration of a stimulus with different patterns of activity (Table III.3) is not compatible with DØving's hypothesis. Thus, there is no compelling evidence to indicate that a particular temporal response pattern represents a particular stimulus quality.

Although a particular response pattern was not elicited to a particular stimulus across different neurons, single olfactory bulb neurons did show different temporal response patterns to different stimuli (Fig. III.6, Table III.3). The results of the present and previous studies (Kauer, 1991; Hamilton and Kauer, 1989; Kauer, 1974; Mac Leod, 1976; Meredith and Moulton, 1978; Meredith, 1986; Reinken and Schmidt, 1986; Wellis et al., 1989; Mair, 1982b) are consistent with an ensemble code, originally proposed for the quality coding of gustatory stimuli (Erickson, 1963). In an ensemble code, molecular

properties of the stimulus are encoded by temporal response patterns of a population of olfactory bulb neurons (Meredith and Moulton, 1978). The ensemble coding scheme is compatible with an "across-fiber" coding scheme in which stimulus quality is encoded by response patterns across a population of neurons (Lancet, 1991; Kauer, 1991) (Chapter II).

CHAPTER IV

RESPONSES OF SINGLE OLFACTORY BULB NEURONS TO BINARY MIXTURES OF AMINO ACIDS

INTRODUCTION

Although chemical mixtures are the stimuli that animals generally encounter in nature, the vast majority of electrophysiological studies within the vertebrate olfactory system tested odorants presented only singly. For a better understanding of the olfactory coding process, however, it is critical to determine how the olfactory system processes neural information concerning stimulus mixtures. Numerous studies, especially those involving crustaceans, indicated that the olfactory system treats mixtures differently than single substances (Derby et al., 1991a; Derby et al., 1991b; Derby and Ache, 1984a; Zimmer-Faust et al., 1984; Derby et al., 1985; Gleeson and Ache, 1985; Johnson et al., 1985; Johnson et al., 1989; Borroni et al., 1986; Carr and Derby, 1986a; Carr and Derby, 1986b; Atema et al., 1989). The difficulty in predicting the responses to stimulus mixtures in the previous studies was attributed to mixture interactions, both mixture suppression and synergism. Previous reports of electro-olfactogram (EOG) and multiunit olfactory receptor responses of channel catfish to binary, trinary and complex mixtures of amino acids, however, clearly indicated that: (a) there was no evidence for mixture suppression, (b) the simultaneous activation of relatively independent receptor sites by the components in the mixture resulted in enhanced olfactory responses, and (c) the responses of olfactory receptors to mixtures were predictable (Caprio et al., 1989; Kang and Caprio, 1991). For result (a), when the stimuli were adjusted in concentration to be equipotent, then mixtures of equal aliquots of these solutions never resulted in suppressed responses.

These data suggested that the majority of the mixture suppression previously reported may have been due to competition between a strong and a weak agonist for specific olfactory receptor sites. The studies that lead to result (b) demonstrated that mixtures whose components showed little cross-adaptation initiated enhanced responses compared with those whose components were indicated to interact with receptor sites having highly overlapping specificities. Thus, the response enhancement that occurred by mixing stimuli that bind to relatively independent receptor sites may be one mechanism of synergism. Result (c) indicated that if knowledge of the responses to the individual components in the mixture and the relative independence of the respective receptors sites for the component stimuli were known, then EOG and integrated neural responses of olfactory receptor cells to mixtures consisting of up to 10 different amino acids were predictable.

With the exception of Chapter II of this dissertation, previous results of how the olfactory system of the channel catfish responded to stimulus mixtures were obtained solely from populations of receptor cells. Understanding how single neurons within the olfactory system respond to stimulus mixtures is essential for a better insight into the olfactory coding process. Mitral cells of the olfactory bulb receive input from olfactory receptor neurons and process and transmit neural information concerning odorants to higher levels within the central nervous system (CNS). Results of Chapter II indicated that no mixture interactions that changed response types of ORNs from those observed to the individual components were encountered, and that the

response types to binary mixtures whose components result in the same response types were predictable.

Since the convergence ratio of vertebrate olfactory receptor neurons to single output neurons of the olfactory bulb is >1000:1 (Satou, 1990), the responses of single olfactory bulb neurons to stimulus mixtures provide a window to a better understanding of the neural processing of mixture information at initial levels within the CNS. Further, the previous results (Chapter III) that responses of olfactory bulb neurons to amino acids were highly reproducible and that the response types to a given stimulus at different concentrations did not change from excitation to suppression, or *vice versa*, provided the background for this study of olfactory bulb neuron responses to binary mixtures of amino acids.

The present study was designed to study: (1) whether olfactory bulb neurons of the channel catfish respond to binary mixtures of amino acids differently than how they respond to the individual components tested separately, (2) whether mixture interactions are evident in the responses of olfactory bulb neurons to binary mixtures of amino acids, and (3) whether it is possible to predict the responses of single olfactory bulb neurons to binary mixtures based on the responses to the individual components. The results indicated that: (a) responses of single olfactory bulb neurons to binary mixtures were highly associated with the responses to the component amino acids, (b) rarely did mixture interactions change response types of olfactory bulb neurons from those observed to the individual components, and (c) responses of single olfactory bulb neurons to

binary mixtures whose components when tested individually resulted in the same response types were generally predictable; where response types to the individually tested components were different, the predictability of the responses was dependent upon the specific mixtures tested.

METHODS AND MATERIALS

Animal preparation, stimulus delivery, electrophysiological methods and data acquisition are identical to those described in the previous paper (Chapter III). Sixty-one olfactory bulb neurons in 36 channel catfish preparations were tested in these binary mixture studies.

Stimuli: Binary Mixtures

Responses of single olfactory bulb neurons to binary mixtures of amino acids were recorded and compared with responses to their corresponding, individually applied component amino acids (Fig. IV.1). To form binary mixtures, two L-amino acids were chosen from eight test amino acids and their concentrations were adjusted to the nearest whole log concentration that elicited similar EOG magnitudes determined in previous mixture experiments (Kang and Caprio, 1991; for example, see Fig. III.6 in Chapter III). Of the eight stimuli, two amino acids from each of the four general categories (acidic, basic, neutral with long side-chain, neutral with short side-chain) were selected. The adjusted concentrations for the test stimuli were 10^{-4} M for Met, nVal, Ala, Gln, Arg, and Lys and 10^{-3} M for Glu and Asp. Binary mixtures were formed by mixing equal aliquots of two single amino acid solutions at twice the concentrations stated above, so that

the resulting concentrations of each of the individual amino acids in the binary mixture would be equal to those tested individually. The pH of all stimulus solutions tested was between 8.0 and 8.6.

Data Analysis

Responses of single olfactory bulb neurons to binary mixtures and to their components were classified as excitatory (E), suppressive (S), or null (N) (see Fig. IV.1 for examples) based on the interrupted time-series analysis (Hudson, 1977); Chapters II and III). The tests were conducted on the number of action potentials elicited by binary mixtures and their components within successive 200-ms time bins during 5-s prestimulation and 5-s stimulation periods.

To explore possible explanations for the different response types to each category (see Results in pages 151-152 for definition) of binary mixtures, percent response changes in the cells' activities during a 5-s stimulation period over that of a 5-s prestimulation period were calculated according to the formula:

$$\text{Percent Response Change (\%)} = 100 \times \frac{\text{POST} - \text{PRE}}{\text{PRE}}$$

where POST and PRE are the number of action potentials occurring during 5-s prestimulation and 5-s stimulation periods, respectively. The mean percent response changes were further analyzed using *t* tests for two-sample cases and ANOVA with SAS (1986, SAS Institute Inc., Cary, NC) for three-sample cases.

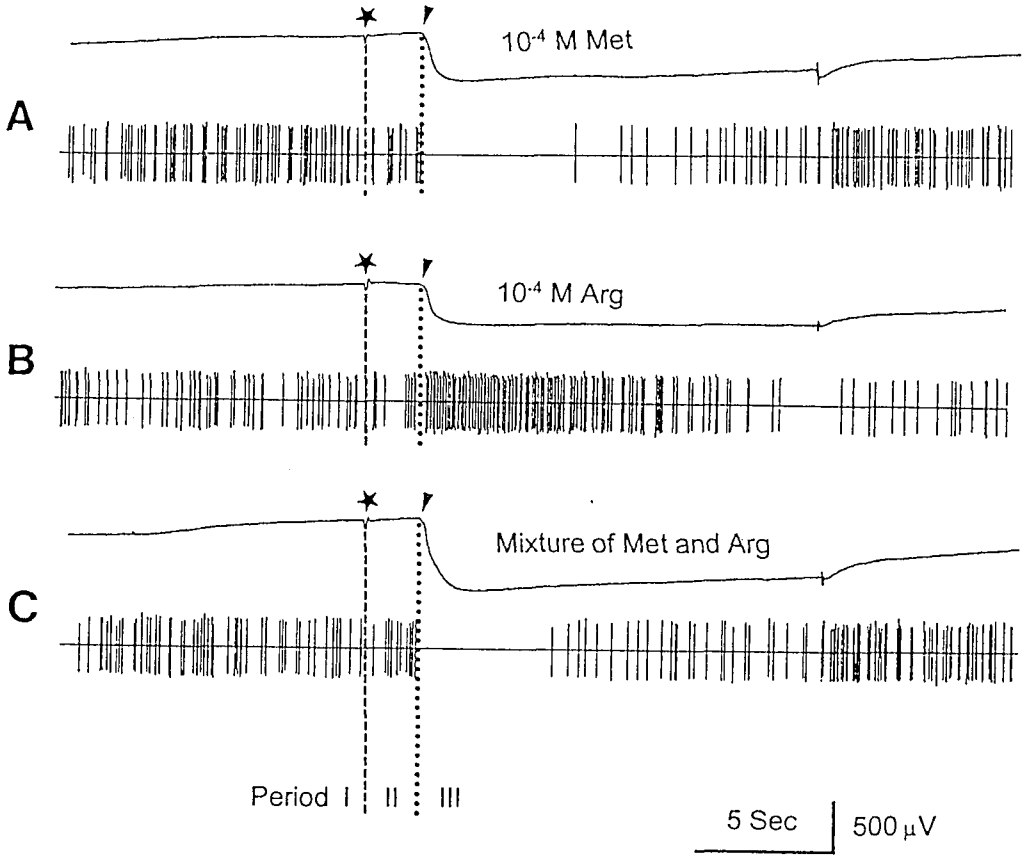
To compare in more detail the similarity in the temporal response patterns, a nonparametric statistical analysis (Spearman Correlation) was performed (Meredith and Moulton, 1978). The number

of action potentials occurring in response to a stimulus (either binary mixtures or their components) within successive 1-s time bins over 10-s prestimulus and 20-s response periods was subjected to the Spearman Correlation tests using SAS. The Spearman Correlation tests determined whether the temporal response patterns to the two components were similar to each other and whether the temporal response pattern to the corresponding mixture was similar to that of either or both components. If the temporal response pattern to the mixture was not similar to that of either component, the response pattern was compared with the average of the responses to the two components.

RESULTS

Responses of olfactory bulb neurons to mixtures and to their components were classified as excitatory (E), suppressive (S), or null (N) based on interrupted time-series analysis of the number of action potentials occurring within successive 200-ms time bins during 5-s prestimulation (Period I, Fig. IV.1) and 5-s stimulation (Period III, Fig. IV.1) periods. Although "null" literally means no significant change from spontaneous activity, for the sake of simplicity in describing the results of the present experiments "null" is defined as a response type (i.e., a "no response"). Binary mixtures whose component responses were similarly classified as both E, both S, and both N were referred to as "component-similar" mixtures. There were three categories of component-similar mixtures: category I (E+E), II (S+S) and III (N+N). Binary mixtures whose component responses were classified differently (i.e., E and N, S and N, and E and S) were

Fig. IV.1. Electro-olfactogram (EOG) (upper traces; negative downward) recorded from the catfish olfactory epithelium and action potentials (lower traces) recorded simultaneously from a single olfactory bulb neuron. (*A*) Suppressive response to 10^{-4} M Met; (*B*) Excitatory response to 10^{-4} M Arg; (*C*) Suppressive response to the corresponding binary mixture of Met and Arg, where each component was at 10^{-4} M in the mixture. The initial small deflection (asterisk) of the EOG record that preceded the onset of the EOG response is an experimental artifact due to the slight pressure pulse created by switching the stimulus injection valve. The onset of the EOG response (arrowhead) indicated the response onset of the olfactory bulb neuron. The neural response trace was divided into three periods, I (prestimulation), II (stimulus delivery) and III (stimulation), for the quantification of the action potentials. Prestimulation and stimulus delivery periods were separated by dashed lines, whereas stimulus delivery and stimulation periods were separated by dotted lines. Action potentials occurring within period II were not used in the analysis of the responses.


A
10^{-4} M Met
B
10^{-4} M Arg
C
Mixture of Met and Arg
Period I II III
5 Sec
500 μV

referred to as "component-different" mixtures. There were three categories of component-different mixtures: category IV (E+N), V (S+N) and VI (E+S).

Chi-square analysis (X^2=208.47, P<0.0001, df=10) indicated that the response types of olfactory bulb neurons to binary mixtures were highly associated with the response types elicited by their component amino acids.

Response Types to Binary Mixtures

Two hundred and ninety-seven mixture trials consisting of 18 different pairs of stimuli (Table IV.1) formed from eight amino acids were presented to the olfactory epithelia in a total of 36 channel catfish. Responses to these mixtures and to their components were recorded from 61 single olfactory bulb neurons. For 126 of 297 (42.4%) tests, no statistically significant responses (N) to the binary mixtures occurred. The responses to the remaining binary mixtures (171 of 297 = 57.6%) were classified as either E (85 of 297 = 28.6%) or S (86 of 297 = 29.0%) types (Table IV.1). Results from Chi-square analysis performed on those mixtures which were tested at least 15 times indicated that the evoked responses were not associated with specific stimulus mixtures (X^2=9.95, P>0.5, df=12).

Component-Similar Binary Mixtures

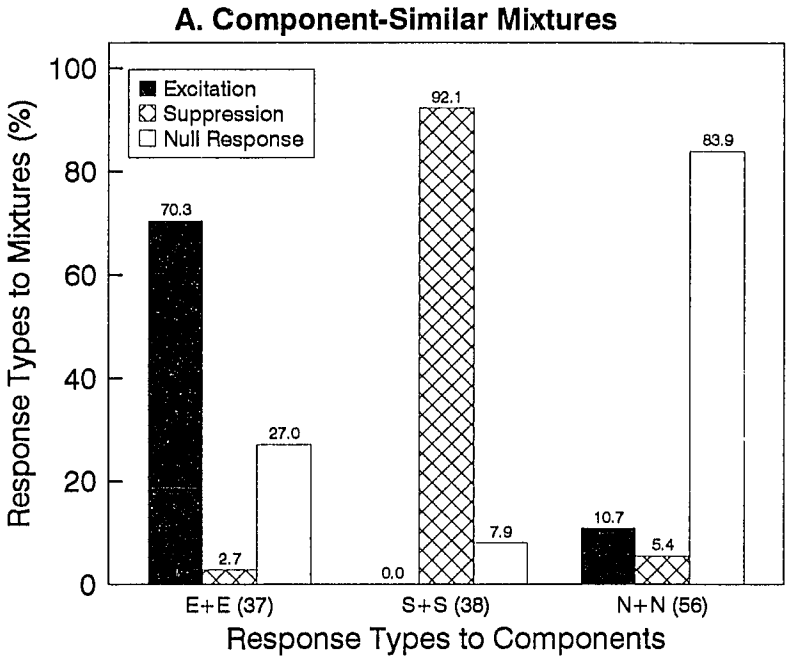
The responses of olfactory bulb neurons to 297 binary mixtures were grouped into 6 categories based on the types of responses elicited by each of the two component amino acids comprising the mixture (Fig. IV.2; Table IV.2). Of these, 131 tests included mixtures whose component responses were similarly classified as both

Table IV.1. *Response Types to Binary Mixtures*

Mixtures	Response Types			Total
	E*	S*	N*	
Met+nVal	16	13	34	63
Met+Ala	4	2	4	10
Met+Arg	11	11	13	35
Met+Lys	5	0	1	6
Met+Glu	7	3	7	17
Met+Asp	2	5	3	10
Ala+Gln	6	5	16	27
Ala+Arg	3	5	3	11
Ala+Lys	0	1	0	1
Ala+Glu	0	3	0	3
Ala+Asp	1	2	2	5
Gln+Glu	0	2	0	2
Arg+Lys	11	14	21	46
Arg+Glu	6	3	6	15
Arg+Asp	0	4	2	6
Lys+Glu	4	0	0	4
Lys+Asp	1	1	1	3
Glu+Asp	8	12	13	33
Total	85	86	126	297

* E, excitatory responses; S, Suppressive responses; N, null.

Fig. IV.2. Summary of responses of olfactory bulb neurons to binary mixtures of amino acids. (*A*) Responses to component-similar mixtures. E+E (37): 37 tests of binary mixtures whose components elicited only excitatory responses; S+S (38): 38 tests of binary mixtures whose components elicited only suppressive responses; N+N (56): 56 tests of binary mixtures whose both components failed to elicit statistically significant responses. (*B*) Responses to component-different mixtures. E+N (63): 63 tests of binary mixtures in which one component elicited an excitatory response and the other component was nonstimulatory (null); S+N (71): 71 tests of binary mixtures in which one component elicited a suppressive response and the other component was nonstimulatory (null); E+S (32): 32 tests of binary mixtures in which one component elicited an excitatory response and the other elicited a suppressive response.


A. Component-Similar Mixtures
Excitation
Suppression
Null Response
Response Types to Mixtures (%)
100
80
60
40
20
0
70.3
2.7
27.0
0.0
92.1
7.9
10.7
5.4
83.9
E+E (37)
S+S (38)
N+N (56)
Response Types to Components

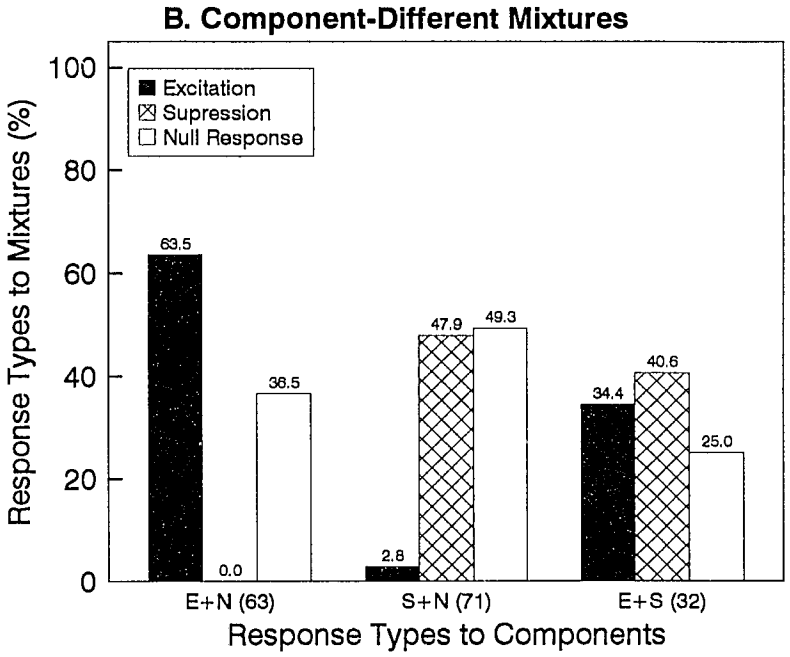

B. Component-Different Mixtures
Excitation
Supression
Null Response
Response Types to Mixtures (%)
100
80
60
40
20
0
63.5
0.0
36.5
2.8
47.9
49.3
34.4
40.6
25.0
E+N (63)
S+N (71)
E+S (32)
Response Types to Components

Table IV.2. *Statistical Analysis of Responses to Binary Mixtures*

Mixture Category		Response Types to MIXTURES (n_1)*	Percent Response Changes (%) to COMPONENTS [Mean±SE (n_2)]**	Statistical Analyses***
A. Component-Similar Binary Mixtures				
I (E+E)		E (26) N (10)	402.9±83.3 (52) 118.7±16.1 (20)	*t* test (df=70, *P*<0.025)
II (S+S)		S (35) N (3)	-72.6±2.4 (70) -57.1±7.8 (6)	*t* test (df=74, *P*<0.05)
III (N+N)		E (6) N (47)	22.7±11.8 (12) 4.4±2.9 (94)	*t* test (df=104, *P*<0.025)
B. Component-Different Binary Mixtures				
IV (E+N)		E (40) N (23)	227.4±47.9 (40) 89.9±18.9 (23)	*t* test (df=61, *P*<0.025)
V (S+N)		S (34) N (35)	-80.2±3.4 (34) -63.2±3.4 (35)	*t* test (df=67, *P*<0.005)
VI (E+S)	*a*	E (11) S (13) N (8)	140.8±14.7 (11) 145.3±25.0 (13) 124.6±16.7 (8)	ANOVA (*P*≥0.79)
	b	E (11) S (13) N (8)	-81.4±4.7 (11) -82.2±5.1 (13) -75.0±5.7 (8)	ANOVA (*P*≥0.61)

* n_1, number of responses to the respective binary mixtures that were so categorized. One suppressive response evoked by E+E mixtures, three suppressive evoked by N+N mixtures and two excitatory responses evoked by S+N mixtures were not included in the statistical analyses due to their small sample sizes with large variances.

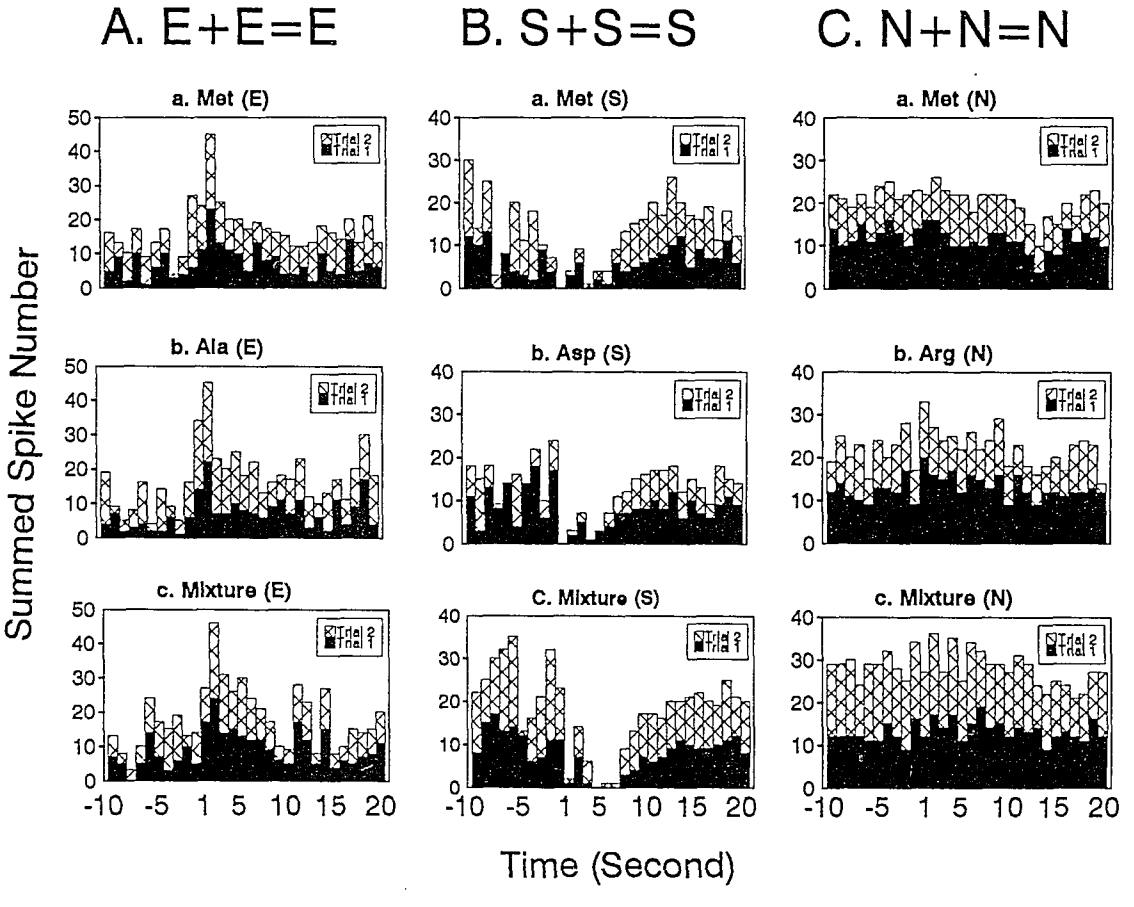
** For E+E, S+S and N+N mixtures, the mean percent response change was the average of the percent response changes caused by both components. Thus, $n_2=2Xn_1$. For E+N and S+N mixtures, the mean percent response change was the average of the percent response changes caused by the E and S components, respectively. Thus, $n_2=n_1$. For E+S mixtures, the mean percent response change was the average of the percent response changes caused by both components, but calculated and listed separately. Thus, $n_2=n_1$.

*** The mean percent response changes were analyzed with one-tailed *t* tests for two-sample cases and with a one-way ANOVA using SAS for three-sample cases.

E, both S, or both N, respectively, and were thus termed "component-similar" mixtures. For these 131 component-similar mixtures, 82.4% resulted in the same response types as their components (Fig. IV.2A).

Category I (E+E). For 37 tests comprising 10 different binary mixtures whose component amino acids evoked excitatory responses, 26 (70.3%) mixtures evoked excitatory responses (Fig. IV.3A), whereas 10 (27.0%) mixtures resulted in null responses and only 1 (2.7%) mixture response was suppressive (Fig. IV.2A). To determine whether the difference in types of responses to the binary mixtures was correlated with (or could be explained by) any differences in the response magnitudes to the components, percent response changes (see Methods and Materials in page #150 for definition) were calculated for the responses to the components. Since both components of mixtures within Category I (E+E) evoked excitatory responses, the percent response changes for both components were calculated. The percent response changes for the components whose mixtures resulted in the same response type (E and N, respectively) were averaged. For the components whose mixtures evoked excitatory responses, the mean percent response change was 402.9±83.3 (mean±SE, n=52), whereas for the components whose mixtures resulted in null responses, the mean percent response change was 118.7±16.1 (n=20) (Table IV.2). Statistical analysis (*t* tests) indicated that although all responses to the components of the binary mixtures in Category I were classified as excitatory, the mean percent response change in the number of action potentials elicited by the component amino acids whose E+E mixtures resulted in excitatory responses was significantly greater

Fig. IV.3. Representative examples of responses of olfactory bulb neurons to component-similar mixtures and to their components. (*A*) Excitatory (E) responses to a binary mixture whose component amino acids were both excitatory (E); (*B*) Suppressive (S) responses to a binary mixture whose component amino acids were both suppressive (S); (*C*) Null (N) responses to a binary mixture whose component amino acids were nonstimulatory. The responses in this figure were obtained from three different olfactory bulb neurons (A, B, C) to two presentations of each stimulus. In this and subsequent histograms, the negative numbers in the time scale denote prestimulation periods. The onset of the stimulation occurred at the start of the one second mark. Bars representing the response to trial two are stacked on top of those for trial one. For example, in histogram *A3* of this figure, the action potential number for trial two occurring within the first second of the stimulation period was 10, i.e., 27 (total) minus 17 (trial one).


A. E+E=E
B. S+S=S
C. N+N=N
a. Met (E)
a. Met (S)
a. Met (N)
b. Ala (E)
b. Asp (S)
b. Arg (N)
c. Mixture (E)
C. Mixture (S)
c. Mixture (N)
Trial 2
Trial 1
Summed Spike Number
Time (Second)
-10 -5 1 5 10 15 20

than that evoked by the components whose E+E mixtures resulted in null responses (Table IV.2).

Category II (S+S). For 38 tests comprising 13 different binary mixtures whose component amino acids evoked suppressive responses (i.e., S+S mixtures), 35 (92.1%) mixtures evoked suppressive responses (Fig. IV.3*B*) and 3 (7.9%) tests resulted in null responses (Fig. IV.2*A*). The mean percent response changes were similarly calculated as for Category I. Statistical analysis (*t* test) indicated that the component amino acids whose S+S mixtures evoked suppressive responses caused a significantly greater mean percent response change in the activity of olfactory bulb neurons (i.e., more suppressed) than that caused by the components whose S+S mixtures resulted in null responses (Table IV.2).

Category III (N+N). For 56 tests comprising eight different binary mixtures whose component amino acids failed to elicit a response (null), 47 (83.9%) mixtures resulted in null responses (Fig. IV.3*C*), whereas six (10.7%) mixtures evoked excitatory and three (5.4%) mixtures evoked suppressive responses (Fig. IV.2*A*). The mean percent response changes were similarly calculated as for Category I. Statistical analysis (*t* test) indicated that although all component responses were classified as null, the mean percent response change in the number of action potentials elicited by the components whose N+N mixtures resulted in excitatory responses was significantly greater than that evoked by components whose N+N mixtures resulted in null responses (Table IV.2). The sample size for suppressive responses to the N+N mixtures was, however, too small (n=3) to determine whether

the mean percent response change caused by the components was significantly different from those caused by the components whose N+N mixtures resulted in excitatory and null responses, respectively.

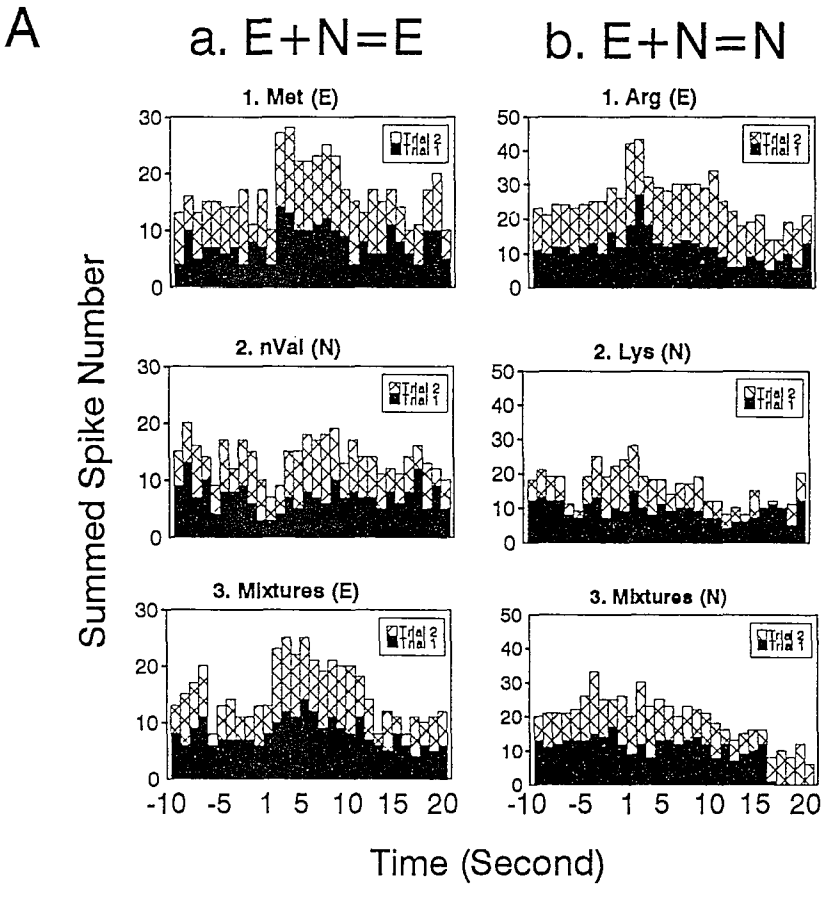
Component-Different Mixtures

One hundred and sixty-six tests were performed with binary mixtures whose component amino acids elicited different types of responses from olfactory bulb neurons (E+N, S+N and E+S) (Fig. IV.2*B*). The results indicated that binary mixtures whose components elicited E or S and N responses, respectively, generally elicited the response type of either of the components and rarely (3%) a response type different than either component.

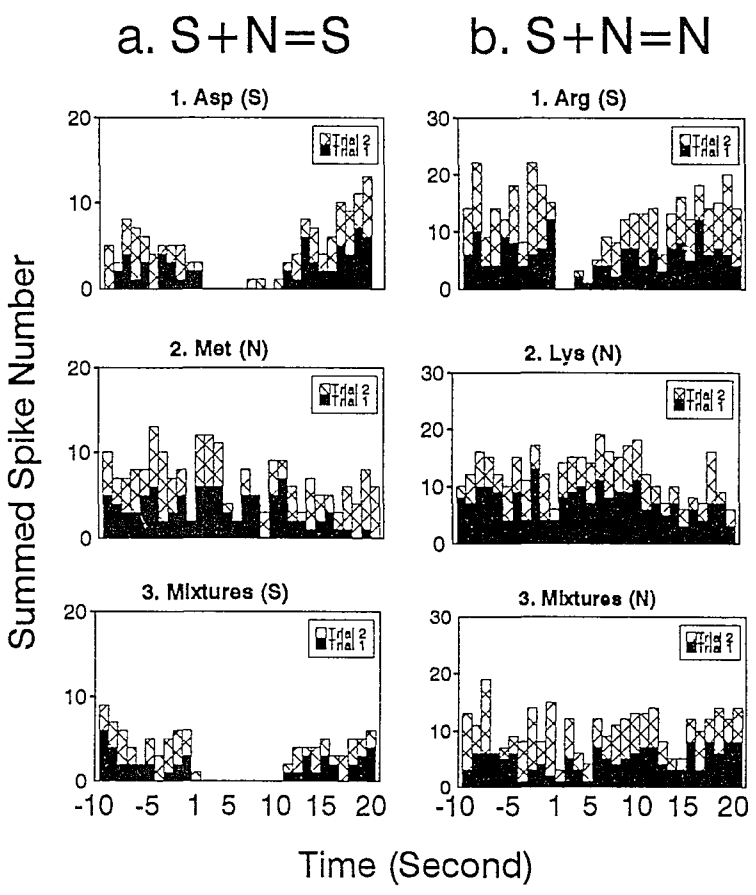
Category IV (E+N). For 63 tests comprising 13 different binary mixtures in which one component evoked an excitatory response and the other component failed to elicit a statistically significant response (E+N), 40 (63.5%) mixture responses were classified as E type and 23 (36.5%) as N type (Figs. IV.2*B* and 4*A*). Since only one of the components evoked excitatory responses, the percent response changes were calculated for the excitatory components only. The mean percent response change for the E components whose E+N mixtures evoked excitatory responses was 227.4±47.9 (mean±se, n=40), which was significantly greater (*t* test) than that (i.e., 89.9±18.9, n=23) for the E components whose E+N mixtures resulted in null responses (Table IV.2).

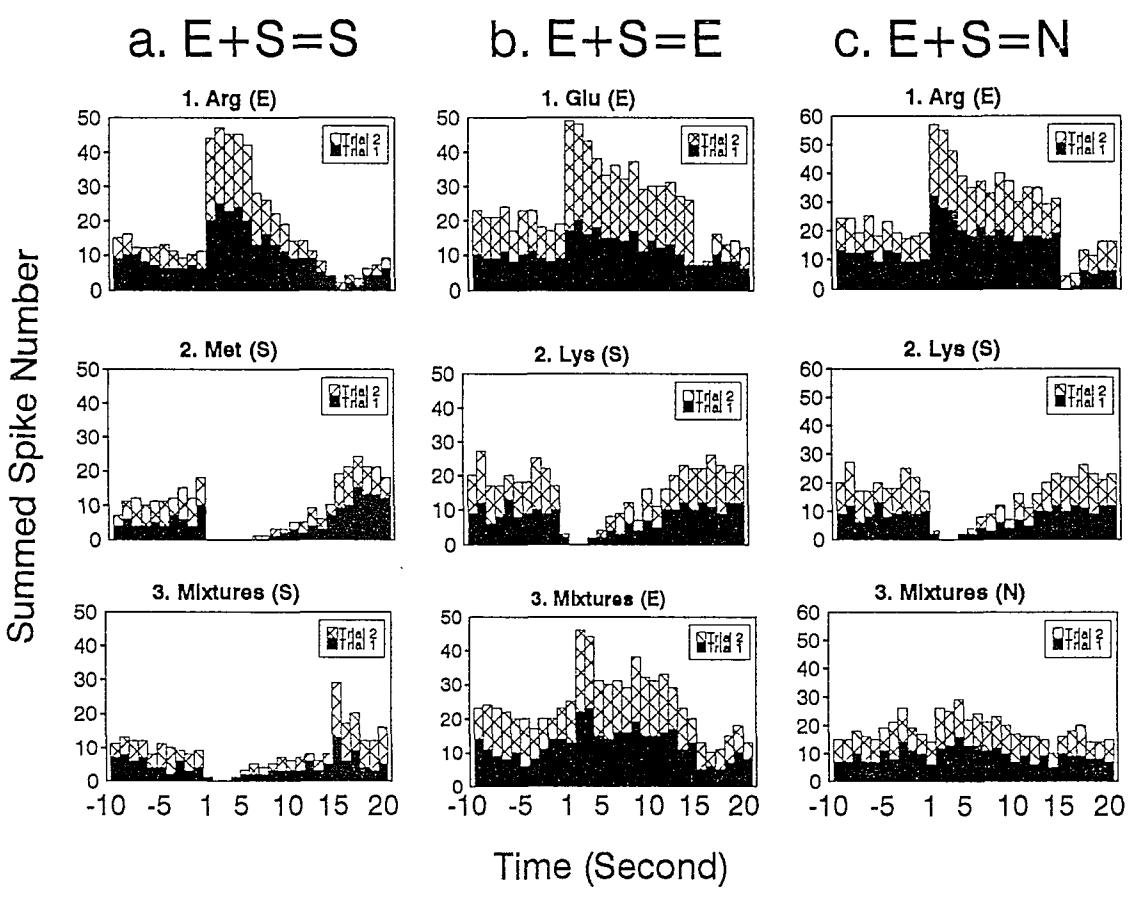
Category V (S+N). For 71 tests comprising 12 different binary mixtures in which one component evoked a suppressive response and the other failed to elicit a statistically significant response (S+N), 34

Fig. IV.4. Representative examples of responses of olfactory bulb neurons to component-different mixtures and to their components. (*A*) E+N binary mixtures elicited from olfactory bulb neurons E (Aa) and N (Ab) responses, respectively. (*B*) S+N binary mixtures elicited S (Ba) and N (Bb) responses, respectively. (*C*) E+S binary mixtures elicited S (Ca), E (Cb) and N (Cc) responses, respectively. The responses in this figure were obtained from six different olfactory bulb neurons (only responses in *Cb* and *Cc* were obtained from same olfactory bulb neuron).


A
a. E+N=E
b. E+N=N
1. Met (E)
1. Arg (E)
2. nVal (N)
2. Lys (N)
3. Mixtures (E)
3. Mixtures (N)
Trial 2
Trial 1
Summed Spike Number
Time (Second)
-10 -5 1 5 10 15 20

B


a. S+N=S
b. S+N=N
1. Asp (S)
1. Arg (S)
2. Met (N)
2. Lys (N)
3. Mixtures (S)
3. Mixtures (N)
Trial 2
Trial 1
Summed Spike Number
Time (Second)
-10 -5 1 5 10 15 20


C
a. E+S=S
b. E+S=E
c. E+S=N
1. Arg (E)
1. Glu (E)
1. Arg (E)
2. Met (S)
2. Lys (S)
2. Lys (S)
3. Mixtures (S)
3. Mixtures (E)
3. Mixtures (N)
Trial 2
Trial 1
Summed Spike Number
Time (Second)
-10 -5 1 5 10 15 20

(47.9%) of the mixture responses were classified as S type, 35 (49.3%) as N type, and 2 (2.8%) as E type (Figs. IV.2*B* and IV.4*B*). Since only one of the components evoked suppressive responses, the percent response changes were calculated for the suppressive components only. The mean percent response change for the S components whose S+N mixtures elicited suppressive responses was significantly greater (i.e., more suppressed; *t* test) than that caused by the S components whose S+N mixtures resulted in N responses (Table IV.2). The sample size for the excitatory responses to the S+N mixtures, however, was too small (n=2) to determine whether the component mean percent response change was significantly different from that caused by the components whose mixtures resulted in suppressive and null responses, respectively.

Category VI (E+S). For 32 tests comprising 13 different binary mixtures in which one component elicited an excitatory response and the other component evoked a suppressive response (i.e., E+S), the following types of responses were evoked: S (40.6%) > E (34.4%) > N (25.0%) (Figs. IV.2*B* and IV.4*C*). Since both components in this category evoked significant (excitatory and suppressive, respectively) responses, the percent response changes for both components were calculated. The percent response change for the components whose binary mixtures resulted in the same response type were averaged separately for the excitatory (a) and suppressive (b) components (Table IV.2). The mean percent response change for the E components whose E+S mixtures evoked excitatory, suppressive and null responses was 140±14.7 (mean±SE, n=11), 145.3±25.0 (n=13) and 124.6±16.7 (n=8),

respectively, whereas that for the S components whose E+S mixtures evoked excitatory, suppressive and null responses was -81.4±4.7 (n=11), -82.2±5.1 (n=13) and -75.0±5.7 (n=8) (Table IV.2). Statistical analysis (ANOVA) was separately performed on the percent response changes caused by E and S components. Results indicated that the mean percent response changes caused by the E and S components, respectively, whose mixtures resulted in E, S, and N responses, respectively, were not significantly different from each other (Table IV.2). For example, there were no significant differences in the mean percent response changes for the E components whose mixtures resulted in E responses than that for the E components whose mixtures resulted in S responses to account for the different response types (i.e., E, S) to the binary mixtures.

Overall, for 166 tests with component-different mixtures, only 10 (6.0%) mixtures elicited a response type that was not observed in the response to one of the component amino acids (Fig. IV.2*B*).

Similarity: Spearman Correlation Tests

Spearman correlation analysis was performed to examine the similarity between the temporal response patterns of olfactory bulb neurons to the two components which were used to form each binary mixture. 110 sets of components, which were used to form a binary mixture, evoked similar temporal response patterns, whereas 187 sets of components evoked different temporal response patterns (Table IV.3). For the 110 binary mixtures whose components elicited similar temporal response patterns, 56.4% of the temporal response patterns evoked by the mixtures were determined to be similar to those evoked

Table IV.3. Comparison (Spearman Correlation) of Temporal Response Patterns of Olfactory Bulb Neurons Between Binary Mixtures and Their Components

Response Patterns to Binary Mixture	Response Patterns to Components	
	Similar to Each Other	Different from Each Other
Similar to Both Response Patterns to the Components	62 (56.4)*	19 (10.2)
Similar to One of Response Patterns to the Components	24 (21.8)	107 (57.2)
Similar to the Average of Response Patterns to the Components	1 (0.9)	13 (7.0)
Similar to None of Response Patterns to the Components	23 (20.9)	48 (25.7)
Total	110 (100)	187 (100)

* Numbers in each column indicate the unit number in that category and the percentages (in parentheses).

by both of their components. Only 21.8% were determined to be similar to those evoked by only one of their component (Table IV.3). In contrast, for the 187 binary mixtures whose components elicited different temporal response patterns, 57.2% of the temporal response patterns evoked by the mixtures were determined to be similar to those evoked by only one of their components, and only 10.2% were determined to be similar to those evoked by both components (Table IV.3).

DISCUSSION

Mixture Interactions and Predicting the Responses to Binary Mixtures

Mixture interactions have been operationally defined as chemosensory phenomena which cause the unpredictability of the response to a mixture with knowledge of the responses to its components (Derby et al., 1989; Bartoshuk, 1975; Hyman and Frank, 1980). The most frequently cited mixture interactions for both olfactory and taste responses are mixture suppression and synergism (Derby and Ache, 1984a; Bartoshuk and Gent, 1985; Johnson et al., 1989; Carr and Derby, 1986a; Carr and Derby, 1986b). Physiological studies of olfaction (Derby et al., 1991a; Derby et al., 1985; Ache, 1989; Derby and Ache, 1984a; Derby and Ache, 1984b) and psychophysical studies of both olfaction (Laing and Glemarec, 1992; Bell et al., 1987; Cain, 1975; Gillan, 1983) and taste (Gillan, 1982; Kroeze, 1982; Lawless, 1979) have suggested that mixture interactions were not only expressed, but were also generated at both peripheral and central neural levels in the olfactory system. In contrast, olfactory receptor responses to binary, trinary (Caprio et al., 1989) and more complex (Kang and Caprio, 1991) mixtures in channel catfish indicated

that there was no evidence for mixture suppression at the peripheral neural level. Additionally, these later reports suggested a mechanism for synergism was the simultaneous activation of relatively independent receptor sites by the components in the mixture. Both reports on olfactory receptor responses to amino acid mixtures in channel catfish were consistent in indicating that it was possible to predict the magnitude of the electro-olfactogram and integrated neural responses to stimulus mixtures based on the responses to the individual components if the relative independence of the respective olfactory receptor sites for the component stimuli in the mixtures were known. However, prior to the present study, no electrophysiological investigation of the responses of single neurons to odor mixtures within central neural olfactory pathways in any vertebrate had been reported.

Component-Similar Mixtures. Chapter II showed that 100% of the responses of single olfactory receptor neurons to component-similar binary mixtures (i.e, S+S and N+N) were similar to the responses evoked by their components. These results indicated that mixture interactions at the single olfactory receptor cell level were rare for component-similar binary mixtures and that the response types were predictable. The present results showed that the majority (82%) of the responses of the single olfactory bulb neurons recorded to component-similar binary mixtures were also similar to the responses to their components (Figs. IV.2A and IV.3). These results were consistent with those obtained from olfactory receptor neurons in the channel catfish and indicated that mixture interactions for component-

similar mixtures were rare for olfactory bulb neurons. Statistical analyses (*t* tests) indicated that the mean percent changes in the number of action potentials elicited by the components whose binary mixtures (i.e., E+E, S+S and N+N) resulted in significant change from background responses were significantly greater than those evoked by the components whose mixtures resulted in null activity (Table IV.2). Thus, by calculating the mean percent changes of response magnitudes to the components, the response types of olfactory bulb neurons to "component-similar" mixtures were generally predictable.

The neural mechanisms responsible for the remaining 19% of the tests in which the E+E and S+S mixtures resulted in different response types than those to either components are currently unknown; however, mixture interactions at both central and peripheral neural levels might have contributed to the unpredictability of the responses. One central neural mechanism that could account for the unexpected null responses to E+E and S+S mixtures might simply be the intensity-response functions of single olfactory bulb neurons. Some single olfactory bulb neurons evoked E or S type response to low stimulus concentrations, but failed to respond to higher stimulus concentrations (Figs. III.9Ac and III.9Ab; Table III.5, Chapter III). The experimental results in the present study showing that more cases of null activity occurred to E+E mixtures (10 of 37) than to S+S mixtures (3 of 38) (Fig. IV.2A) were consistent with the results of Chapter III. The response properties of single olfactory bulb neurons to individual amino acids suggested that olfactory bulb neurons were more likely to respond with N responses to E+E mixtures than to S+S

mixtures since more intensity-response functions changed from E to N responses (7 of 16) to a given stimulus with increasing concentrations than those which changed from S to N responses (2 of 16) (Table III.5). However, the mean percent response change for (1) the components whose E+E mixtures evoked excitatory, and (2) the components whose S+S mixtures evoked suppressive responses were of significantly greater magnitude than those for the components whose mixtures resulted in null responses (Table IV.2). These results support the possibility that the 19% of the unpredicted responses to the E+E and S+S mixtures might at least partially result from peripheral competitive (Michel et al., 1991; Gleeson and Ache, 1985; Ache et al., 1988; McClintock and Ache, 1989; Ache, 1989; Derby et al., 1991a) and/or noncompetitive (Laing and Glemarec, 1992; Bell et al., 1987) mechanisms of inhibition.

Although the components when tested individually could fail to stimulate an olfactory bulb neuron, the response of that same neuron to the binary mixture could be either excitatory or suppressive. In the present experiments, 11% of the responses to N+N mixtures were excitatory and 5% were suppressive (Fig. IV.2A). A possible peripheral neural mechanism that could be responsible for these results is the spatial summation of subthreshold responses to components of a binary mixture acting on two independent sites occurring on the same olfactory receptor neurons (Chapter II; (Ivanova and Caprio, 1993). Also, the combination of two amino acids that interact with different receptor sites, but having some overlapping specificities (Kang and Caprio, 1991; Imamura et al., 1992), might

result in effectively increasing the stimulus concentration of one of the components producing a suprathreshold stimulus. The anatomical convergence of 1000:1 olfactory receptor neurons onto single mitral cells (Satou, 1990) could also be the basis of an additional mechanism accounting for the excitatory and suppressive responses to N+N mixtures. In this case, a binary mixture whose components individually resulted in a response slightly but not significantly different from the background activity of a single ORN, upon convergence with similar responding neurons onto a mitral cell might result in a statistically significant E or S mitral cell response due to the high convergence ratio.

Component-Different Mixtures. With but two exceptions (3%) in which S+N mixtures evoked excitation, responses of single olfactory bulb neurons to E+N and S+N mixtures were similar to that to one of the components (Fig. IV.2B). For 64% of the tests of E+N mixtures and 48% of the tests of S+N mixtures, excitatory and suppressive responses, respectively, were evoked from single olfactory bulb neurons (Figs. IV.2B, 4IV.Aa and 4IV.Ba). Thus, for 36% of the tests of the E+N mixtures and 49% of the tests of the S+N mixtures, the N components effectively masked the effectiveness of the E and S components, respectively (Figs. IV.2B, IV.4Ab and IV.4Bb). Studies of brain interneurons of spiny lobster (Derby et al., 1985; Ache, 1989; Derby and Ache, 1984a) and deuocerebral neurons of potato beetle (De Jong, 1988) indicated that compounds which individually did not evoke excitatory responses from the respective neurons suppressed the efficiency of excitatory components when combined to form mixtures.

However, in the present experiments, the E+N and S+N mixtures that elicited E and S type responses, respectively, contained components that when tested individually were significantly more excitatory and more suppressive, respectively, than the components comprising the E+N and S+N mixtures that resulted in null activity (Table IV.2). Thus, a "null" component can mask either a weak excitatory or a weak suppressive component in a binary mixture, but has little effect on strongly excitatory or strongly suppressive components. It should also be noted that the term "mixture masking" used in the present study has a broader meaning than the term "mixture suppression" in previous studies (Derby et al., 1985; Ache, 1989; Boeckh, 1976; Derby and Ache, 1984a). In "mixture suppression", one component in a mixture only suppressed the neuron's excitatory response to the other component, but in "mixture masking", a component reduces or conceals the neuron's excitatory or suppressive response to the other component.

Comparison of olfactory receptor and bulbar responses to E+N and S+N mixtures suggested that the masking effect of the N component observed at the level of receptor neurons (Fig. II.7) contributed to the mixture masking observed at the level of bulb neurons (Fig. IV.2B); e.g., 11% of the olfactory receptor neurons tested with E+N mixtures and 56% tested with S+N mixtures showed null activity (Chapter II). Thus, the mixture masking effect observed at the level of single olfactory bulb neurons might have resulted from the previously indicated peripheral mechanisms of competitive or noncompetitive inhibition. However, the result that there were more

olfactory bulb neurons (36%, Fig. IV.2B) than receptor neurons (11%, Fig. II.7, Chapter II) showing null activity to E+N mixtures suggested that the majority of the mixture interactions accounting for the masking effect of the N components was due to a central nervous system mechanism. Mixture interactions within the central nervous system might be generated through lateral inhibition between mitral cells via granule cells (Satou, 1990).

For E+S mixtures which resulted in all three possible types of responses, the responses to the mixtures were unpredictable. For these mixtures, no significant differences occurred among the mean percent changes in the response magnitudes caused by the components (Table IV.2). In 25% (8 of 32) of the tests of the E+S mixtures, responses of single olfactory bulb neurons were classified as null. These results indicated that mixture interactions did occur, but they resulted in a simple, predictable cancellation of the E response by the S component. Unfortunately, no data are yet available concerning responses of single olfactory receptor neurons to E+S binary mixtures in the channel catfish that might possibly suggest peripheral neural mechanisms accounting for this response cancellation. However, in the spiny lobster, depolarizing olfactory receptor potentials to L-arginine and L-cysteine were cancelled after combining these stimuli (in a trinary mixture) with L-proline, which by itself evoked a hyperpolarizing response from the same receptor cell (Michel and Ache, 1994). Recent cell-attached, patch clamp recordings of isolated olfactory receptor neurons in the channel catfish indicated that individual amino acids could suppress the spontaneous activity of the

cell; however, hyperpolarizing receptor potentials were never observed in direct response to amino acid stimulation of these isolated receptor neurons in whole cell recording mode. Instead, the evidence indicated that particular amino acids could modulate the activity of voltage-gated potassium channels which might account for the suppression of the neural activity of the cell (Ivanova and Caprio, 1993). Thus, peripheral neural mechanisms may play some role in the observed mixture suppression observed at the level of single olfactory bulb neurons in the channel catfish.

For the other 75% (24 of 32) of the tests of E+S binary mixtures, olfactory bulb neurons responded with either excitation (n=13; 54%) or suppression (n=11; 46%) (Figs. IV.2B and IV.4C), indicating that the responses of one component were masked by the other component. Possible mechanisms for this mixture masking effect for E+S mixtures might be peripheral competitive or noncompetitive inhibition, and/or central lateral inhibition, similar to the mechanisms for the masking effects of the N components in E+N and S+N mixtures.

Binary Mixtures: a New Quality?

The question of whether chemical mixtures are a new quality independent of the quality of their components is a fundamental, but still unresolved issue in olfactory coding (Akers, 1993). Electrophysiological and behavioral studies suggested that due to mixture interactions olfactory systems of crustaceans perceive mixtures as different qualities than their components (Fine-Levy and Derby, 1991; Derby et al., 1991a); (Derby et al., 1991b). However,

the results of the present study suggest that although mixture interactions occur, binary mixture information is processed by olfactory bulb neurons as the same or as a similar quality as that for at least one of the mixture components. Evidence for this conclusion includes: (1) an overall 89% of the responses of single olfactory bulb neurons to the tested binary mixtures were similarly classified as at least one of the stimulus components (Tables IV.2A and IV.2B); (2) the responses of single olfactory bulb neurons to binary mixtures were highly correlated with the responses to their components (Chi-square analysis); (3) 71% of the temporal response patterns to mixtures were similar to that of at least one of the components of binary mixtures (Spearman correlation analysis; Table IV.3). That the qualities of the components are not lost in a binary mixture is in agreement with (1) behavioral studies in honeybees which suggested that olfactory mixtures did not appear to be completely distinct from the qualities of the components (Getz and Smith, 1987; Getz and Smith, 1990), and (2) previous human psychophysical studies which indicated that although features of one or both components may be masked, most of the qualities of both odorants were perceived when odorants were of approximately equal intensity (Laing and Willcox, 1983).

SUMMARY

Electro-olfactogram (EOG) and multiunit olfactory receptor responses to mixtures composed of neutral and acidic or basic amino acids were significantly enhanced compared with those to mixtures consisting of an equal number of only neutral amino acids. These results indicate that receptor sites for the acidic and basic amino acids are independent of those for the neutral amino acids. The increasing magnitude of the EOG and multiunit receptor responses to mixtures consisting of an increasing number of acidic (up to 2), basic (up to 2), and neutral (up to 9) amino acids, respectively, suggested that multiple receptor site types with overlapping specificities exist to amino acids within each group. That the majority of single olfactory receptor neurons responded to different stimuli with different response patterns indicated that multiple receptor site types for different odorants exist within the plasma membranes of individual olfactory receptor neurons. The conclusions that different receptor molecules are specific for different amino acids and that different receptor proteins are coexpressed on single receptor neurons support the hypothesis that stimulus quality is coded by the patterns of activity across populations of olfactory receptor neurons, i.e., an "across-fiber" coding scheme.

Both olfactory receptor and olfactory bulb neurons responded with either facilitation or suppression of their spontaneous activities to odorant stimulation. Suppressive responses to individual odorants were encountered as commonly as excitatory

responses in both peripheral receptor and central olfactory bulb neurons, suggesting that both types of responses are involved in the coding of stimulus quantity and quality. A sensory system having neurons which can be modulated by either excitation or suppression can encode more information concerning odorant stimuli than a system with the same number of receptor neurons which can only be excited.

Results of EOG and multiunit olfactory receptor responses to complex mixtures of amino acids confirmed recent findings that there was no evidence for mixture suppression and that a mechanism for synergism was the simultaneous activation of relatively independent receptor sites by the components in the mixture (Caprio et al., 1989). It was also demonstrated that the neural responses of populations of ORNs to complex mixtures consisting of up to 10 different amino acids were predictable with knowledge of the responses to the individual components in the mixture and the relative independence of the respective receptor sites for the component stimuli.

Results of responses of single olfactory receptor and olfactory bulb neurons to binary mixtures of amino acids indicated that for binary mixtures whose components resulted in similar response types (E+E, S+S or N+N), mixture interactions were rarely observed. Thus, it was generally possible to predict the response types of both olfactory receptor and olfactory bulb neurons to component-similar binary mixtures (i.e., E+E, S+S and N+N). For binary mixtures whose components resulted in different response types (E+N, S+N, or E+S) the results indicated that mixture interactions did occur at both peripheral and central neural levels in the olfactory system of

channel catfish. Nevertheless, mixture interactions that changed response types from those observed to the individual components were rare. These results suggested that the individual quality of the components is not lost in a binary mixture, and that odorant mixtures are processed by the olfactory system of channel catfish as the same quality as that for at least one of the components. Although some mixture interactions were observed in responses of olfactory receptor and bulb neurons to odorant mixtures, it is likely that major fundamental differences in the processing of single odorant and mixture information by olfactory receptor and bulb neurons in the channel catfish do not occur.

REFERENCES

Ache, B. W., Gleeson, R. A. and Thompson, H. A. Mechanisms for mixture suppression in olfactory receptors of the spiny lobster. *Chem. Senses* 13:425-434, 1988.

Ache, B. W. Central and peripheral bases for mixture suppression in olfaction: a crustacean model. In: *Perception of complex smells and tastes*, edited by D. G. Laing, W. S. Cain, R. L. McBride and B. W. Ache. San Diego: Academic Press, 1989, p. 101-114.

Akers, R. P. A test of identified response classes among olfactory receptor neurons in the honey-bee worker. *Chem. Senses* 17:191-209, 1992.

Akers, R. P. Response of olfactory receptor neurons in honeybees to odorants and their binary mixtures. *J. Comp. Physiol. [A]* 173:169-185, 1993.

Atema, J., Borroni, P., Johnson, B., Voigt, R. and Handrich, L. Adaptation and mixture interactions in chemoreceptor cells: mechanisms for diversity and contrast enhancement. In: *Perception of complex smells and tastes*, edited by D. G. Laing, W. S. Cain, R. L. McBride and B. W. Ache. San Diego: Academic Press, 1989, p. 83-100.

Bartoshuk, L. M. Taste mixtures: is mixture suppression related to compression? *Physiol. Behav.* 14:643-649, 1975.

Bartoshuk, L. M. Psychophysical studies of taste mixtures. In: *Olfaction and Taste VI*, 1977, p. 377-384.

Bartoshuk, L. M. and Cleveland, C. T. Mixtures of substances with similar tastes: a test of a psychophysical model of taste mixture interactions. *Sens. Processes* 1:177-186, 1977.

Bartoshuk, L. M. and Gent, J. F. Taste mixtures: an analysis of synthesis. In: *Taste, Olfaction,and the Central Nervous System*, edited by D. W. Pfaff. New York: The Rockefeller University Press, 1985, p. 210-232.

Baylin, F. Temporal patterns and selectivity in the unitary responses of olfactory receptors in the tiger salamander to odor stimulation. *J. Gen. Physiol.* 74:17-36, 1979.

Bell, G. A., Laing, D. G. and Panhuber, H. Odour mixture suppression: evidence for a peripheral mechanism in human and rat. *Brain Res.* 426:8-18, 1987.

Blank, D. L. and Mozell, M. M. Olfactory receptor response characteristics: A factor analysis. *Brain Res.* 1:185-192, 1976.

Bodznick, D. A. Characterization of olfactory bulb units of sockeye salmon with behaviorally relevant stimuli. *J. Comp. Physiol. [A]* 127:147-155, 1978.

Boeckh, J. Inhibition and excitation of single insect olfactory receptors, and their role as a primary sensory code. *Olfaction and Taste II* 721-735, 1976.

Boeckh, J., Ernst, K., Sass, H. and Waldow, U. Anatomical and physiological characteristics of individual neurons in the central antennal pathway of insects. *J. Insect Physiol.* 30:15-26, 1984.

Boeckh, J. and Boeckh, V. Threshold and odor specificity of pheromone-sensitive neurons in the deutocerebrum of Antheraea pernyi and A. polyphemus (Saturnidae). *J. Comp. Physiol. [A]* 132:235-242, 1979.

Borroni, P. F., Handrich, L. S. and Atema, J. The role of narrowly tuned taste cell populations in lobster (Homarus americanus) feeding behavior. *Behav. Neurosci.* 100:206-212, 1986.

Brown, S. B. and Hara, T. J. Accumulation of chemostimulatory amino acids by a sedimentable fraction isolated from olfactory rosettes of rainbow trout (Salmo gairdneri). *Biochim. Biophys. Acta* 675:149-162, 1981.

Bruch, R. C. and Kalinoski, D. L. Interaction of GTP-binding regulatory proteins with chemosensory receptors. *J. Biol. Chem.* 262:2401-2404, 1987.

Bruch, R. C. and Rulli, R. D. Ligand binding specificity of a neutral L-amino acid olfactory receptor. *Comp. Biochem. Physiol. [B]* 91:535-540, 1988.

Buonviso, N. and Chaput, M. A. Response similarity to odors in olfactory bulb output cells presumed to be connected to the same glomerulus: Electrophysiological study using simultaneous single-unit recordings. *J. Neurophysiol.* 64(3):447-453, 1990.

Byrd, R. P.,Jr. and Caprio, J. Comparison of olfactory receptor (EOG) and bulbar (EEG) responses to amino acids in the catfish, Ictalurus punctatus. *Brain Res.* 249:73-80, 1982.

Cagan, R. H. and Zeiger, W. N. Biochemical studies of olfaction: binding specificity of radioactivity labeled stimuli to an isolated olfactory preparation from rainbow trout (Salmo gairdneri). *Proc. Natl. Acad. Sci. USA* 75:4679-4683, 1978.

Cain, W. S. Odor intensity: mixtures and masking. *Chem. Senses Flav.* 1:339-352, 1975.

Cameron, A. T. The taste sense and the relative sweetness of sugars and other sweet substances. *Sci. Rep. Ser.* 9:1-72, 1947.

Cancalon, P. F. Isolation and characterization of the olfactory epithelial cells of the catfish. *Chem. Senses Flav.* 8:381-396, 1978.

Caprio, J. Electrophysiological distinctions between the taste and smell of amino acids in catfish. *Nature* 266:850-851, 1977.

Caprio, J. Olfaction and taste in the channel catfish: an electrophysiological study of the responses to amino acids and derivatives. *J. Comp. Physiol. [A]* 123:357-371, 1978.

Caprio, J. Similarity of olfactory receptor responses (EOG) of freshwater and marine catfish to amino acids. *Can. J. Zool.* 58:1778-1784, 1980.

Caprio, J. Olfaction and taste in fish. In: *Comparative Physiology of Sensory Systems*, edited by L. Bolis, R. D. Keynes and S. M. P. Maddrell. New York: Cambridge University Press, 1984, p. 257-283.

Caprio, J., Dudek, J. and Robinson II, J. J. Prediction of olfactory responses to stimulus mixtures by cross-adaptation experiments. In: *Olfaction and Taste IX*, edited by S. D. Roper and J. Atema. New York: The New York Academy of Sciences, 1987, p. 216-218.

Caprio, J. Olfactory receptor responses to binary mixtures of amino acids in the channel catfish, Ictalurus punctatus. In: *The Beidler Symposium on Taste and Smell, A Festschrift to Lloyd M. Beidler*, edited by Jr. Miller,I.J.. Winston-Salem: Book Service Associates, 1988a, p. 105-114.

Caprio, J. Peripheral filters and chemoreceptor cells in fishes. In: *Sensory biology of aquatic animals*, edited by J. Atema, R. R. Fay, A. N. Popper and W. N. Tavolga. NY: Springer-Verlag, 1988b, p. 313-338.

Caprio, J., Dudek, J. and Robinson, J. J.,II Electro-olfactogram and multiunit olfactory receptor responses to binary and trinary mixtures of amino acids in the channel catfish, Ictalurus punctatus. *J. Gen. Physiol.* 93:245-262, 1989.

Caprio, J. Chemoreception of amino acids in teleosts: peripheral mechanisms of signal processing. *Proc. 24th Jpn. Symp. Taste & Smell* 24:5-9, 1990.

Caprio, J. and Byrd, R. P.,Jr. Electrophysiological evidence for acidic, basic, and neutral amino acid olfactory receptor sites in the catfish. *J. Gen. Physiol.* 84:403-422, 1984.

Caprio, J. and Raderman-Little, R. Scanning electron microscopy of the channel catfish olfactory lamellae. *Tissue Cell* 10(1):1-9, 1978.

Carr, W. E. S. and Derby, C. D. Behavioral chemoattractants for the shrimp, Palaemonetes pugio: identification of active components in food extracts and evidence of synergistic mixture interactions. *Chem. Senses* 11:49-64, 1986a.

Carr, W. E. S. and Derby, C. D. Chemically stimulated feeding behavior in marine animals Importance of chemical mixtures and involvement of mixture interactions. *J. Chem. Ecol.* 12:989-1011, 1986b.

Chaput, M. and Holley, A. Spontaneous activity of olfactory bulb neurons in awake rabbits, with some observations on the effects of pentobarbital anaesthesia. *J. Physiol. (Paris)* 75:939-948, 1979.

Chaput, M. A. and Holley, A. Responses of olfactory bulb neurons to repeated odor stimulations in awake freely-breathing rabbits. *Physiol. Behav.* 34:249-258, 1985.

Chaput, M. A. and Lankheet, M. J. Influence of stimulus intensity on the categories of single-unit responses recorded from olfactory bulb neurons in awake freely-breathing rabbits. *Physiol. Behav.* 40:453-462, 1987.

De Jong, R. and Visser, J. H. Specificity-related suppression of responses to binary mixtures in olfactory receptors of the Colorado potato beetle. *Brain Res.* 447:18-24, 1988.

De Jong, R. Integration of Olfactory Information in the Colorado Potato Beetle Brain. *Brain Res.* 447:10-17, 1988.

Den Otter, C. J. Single sensillum responses in the male moth Adoxophyes orana (F.v.R.) to female sex pheromone components and their geometrical isomers. *J. Comp. Physiol. [A]* 121:205-222, 1977.

Derby, C. D., Hamilton, K. A. and Ache, B. W. Processing of olfactory information at three neuronal levels in the spiny lobster. *Brain Res.* 300:311-319, 1984.

Derby, C. D., Ache, B. W. and Kennel, E. W. Mixture suppression in olfaction: electrophysiological evaluation of the contribution of peripheral and central neural components. *Chem. Senses* 10:301-316, 1985.

Derby, C. D., Girardot, M. -N., Daniel, P. C. and Fine-Levy, J. B. Olfactory discrimination of mixtures: behavioral, electrophysiological and theoretical studies using the spiny lobster Panularis argus. In: *Perception of Complex Smells and Tastes*, edited by D. G. Laing, W. S. Cain, R. L. McBride and B. W. Ache. Sydney: Academic press, 1989, p. 65-81.

Derby, C. D., Girardot, M. -N. and Daniel, P. C. Responses of olfactory receptor cells of spiny lobsters to binary mixtures. I. Intensity mixture interactions. *J. Neurophysiol.* 66:112-130, 1991a.

Derby, C. D., Girardot, M. -N. and Daniel, P. C. Responses of olfactory receptor cells of spiny lobsters to binary mixtures. II. Pattern mixture interactions. *J. Neurophysiol.* 66:131-139, 1991b.

Derby, C. D. and Ache, B. W. Electrophysiological identification of the stimulatory and interactive components of a complex odorant. *Chem. Senses* 9:201-218, 1984a.

Derby, C. D. and Ache, B. W. Quality coding of a complex odorant in an invertebrate. *J. Neurophysiol.* 51:906-924, 1984b.

Derby, C. D. and Atema, J. Chemoreceptor cells in aquatic invertebrates: peripheral mechanisms of chemical signal processing in decapod crustaceans. In: *Sensory biology of aquatic animals*, edited by J. Atema, A. N. Popper, R. R. Fay and W. N. Tavolga. New York: Springer Verlag, 1988, p. 365-385.

Dionne, V. E. Chemosensory responses in isolated olfactory receptor neurons from Necturus maculosus. *J. Gen. Physiol.* 99:415-433, 1992.

Duchamp, A., Revial, M. F., Holley, A. and Mac Leod, P. Odor discrimination by frog olfactory receptors. *Chem. Senses Flav.* 1:213-233, 1974.

Duchamp, A. Electrophysiological responses of olfactory bulb neurons to odour stimuli in the frog. A comparison with receptor cells. *Chem. Senses* 7:191-210, 1982.

Duchamp, A. and Sicard, G. Influence of stimulus intensity on odour discrimination by olfactory bulb neurons as compared with receptor cells. *Chem. Senses* 8:355-366, 1984a.

Duchamp, A. and Sicard, G. Odour discrimination by olfactory bulb neurons: Statistical analysis of electrophysiological responses and comparison with odour discrimination by receptor cells. *Chem. Senses* 9(1):1-14, 1984b.

Duchamp-Viret, P., Duchamp, A. and Vigouroux, M. Amplifying role of convergence in olfactory system a comparative study of receptor cell and second-order neuron sensitivities. *J. Neurophysiol.* 61:1085-1094, 1989.

Duchamp-Viret, P., Duchamp, A. and Vigouroux, M. Temporal aspects of information processing in the first two stages of the frog olfactory system: Influence of stimulus intensity. *Chem. Senses* 15:349-365, 1990.

DØving, K. B. Studies of the relation between the frog's electro-olfactogram (EOG) and single unit activity in the olfactory bulb. *Acta Physiol. Scand.* 60:150-163, 1964.

DØving, K. B. Efferent influence upon the activity of single neurons in the olfactory bulb of the burbot. *J. Neurophysiol.* 29:675-683, 1966.

DØving, K. B., Selset, R. and Thommesen, G. Olfactory sensitivity to bile acids in salmonid fishes. *Acta Physiol. Scand.* 108:123-131, 1980.

DØving, K. B. Response properties of neurons in the rat olfactory bulb to various parameters of odour stimulation. *Acta Physiol. Scand.* 130:285-298, 1987.

DØving, K. B. and Holmberg, K. A note on the function of the olfactory organ of the hagfish Maxine glutinosa. *Acta Physiol. Scand.* 91:430-432, 1974.

Erickson, J. R. and Caprio, J. The spatial distribution of ciliated and microvillous olfactory receptor neurons in the channel catfish is not matched by a differential specificity to amino acid and bile salt stimuli. *Chem. Senses* 9:127-141, 1984.

Erickson, R. P. Sensory neural patterns and gustation. In: *Olfaction and Taste*, edited by Y. Zotterman. Oxford: Pergmon Press, 1963, p. 205-213.

Evans, R. E. and Hara, T. J. The characteristics of the electro-Olfactogram (EOG): Its loss and recovery following olfactory nerve section in rainbow trout (Salmo gairdneri). *Brain Res.* 330:65-75, 1985.

Fine-Levy, J. B. and Derby, C. D. Effects of stimulus intensity and quality on discrimination of odorant mixtures by spiny lobsters in an associative learning paradigm. *Physiol. Behav.* 49:1163-1168, 1991.

Finger, T. E. and Böttger, B. Transcellular labeling of taste bud cells by carbocyanine dye (DiI) applied to peripheral nerves in the barbels of the catfish, Ictalurus punctatus. *J. Comp. Neurol.* 302:884-892, 1990.

Fischer, T. and Zippel, H. P. The effects of cryogenic blockade of the centrifugal, bulbopetal pathways on the dynamic and static response characteristics of goldfish olfactory bulb mitral cells. *Exp. Brain Res.* 75:390-400, 1989.

Gesteland, R. C., Lettvin, J. Y., Pitts, W. H. and Rojas, A. Odor specificities of the frog's olfactory receptors. In: *Olfaction and Taste*, edited by Y. Zotterman. Oxford: Pergamon Press Ltd., 1963, p. 19.

Gesteland, R. C., Lettvin, J. Y. and Pitts, W. H. Chemical transmission in the nose of the frog. *J. Physiol. (Lond.)* 181:525-559, 1965.

Gesteland, R. C. Techniques for investigating single unit activity in the vertebrate olfactory epithelium. In: *Methods in Olfactory Research*, edited by D. G. Moulton, A. Turk and J. W. Johnson,Jr.. London : Academic Press, 1975, p. 269-322.

Getchell, T. V. Analysis of unitary spikes recorded extracellularly from frog olfactory receptor cells and axons. *J. Physiol.* 234:533-551, 1973.

Getchell, T. V. Electrogenic sources of slow voltage transients recorded from frog olfactory epithelium. *J. Neurophysiol.* 37:1115-1130, 1974a.

Getchell, T. V. Unitary responses in frog olfactory epithelium to sterically related molecules at low concentrations. *J. Gen. Physiol.* 64:241-261, 1974b.

Getchell, T. V. and Shepherd, G. M. Responses of olfactory receptor cells to step pulses of odour at different concentrations in the salamander. *J. Physiol.* 282:521-540, 1978a.

Getchell, T. V. and Shepherd, G. M. Adaptive properties of olfactory receptors analyzed with odour pulses of varying durations. *J. Physiol.* 282:541-560, 1978b.

Getz, W. M. and Smith, K. B. Olfactory sensitivity and discrimination of mixtures in the honeybee Apis mellifera. *J. Comp. Physiol. [A]* 160:239-245, 1987.

Getz, W. M. and Smith, K. B. Odorant moiety and odor mixture perception in free-flying honey bees (Apis mellifera). *Chem. Senses* 15:111-128, 1990.

Gillan, D. J. Mixture suppression: the effect of spatial separation between sucrose and NaCl. *Percept. Psychophys.* 32:504-510, 1982.

Gillan, D. J. Taste-taste, odor-odor, and taste-odor mixtures: Greater suppression within than between modalities. *Percept. Psychophys.* 33(2):183-185, 1983.

Gleeson, R. A. and Ache, B. W. Amino acid suppression of taurine-sensitive chemosensory neurons. *Brain Res.* 335:99-107, 1985.

Goh, Y. and Tamura, T. The electrical responses of the olfactory tract to amino acids in carp. *Bull. Jpn. Soc. Sci. Fish.* 44:341-344, 1978.

Goh, Y. and Tamura, T. Olfactory and gustatory responses to amino acids in two marine teleosts--red sea bream and mullet. *Comp. Biochem. Physiol. [C]* 66:217-224, 1980.

Goulding, E. H., Ngai, J., Kramer, R. H., Colicos, S., Axel, R., Siegelbaum, S. A. and Chess, A. Molecular cloning and single-channel properties of the cyclic nucleotide-gated channel from catfish olfactory neurons. *Neuron* 8:45-58, 1992.

Hamilton, K. A. and Kauer, J. S. Patterns of intracellular potentials in salamander mitral/tufted cells in response to odor stimulation. *J. Neurophysiol.* 62(3):609-625, 1989.

Hara, T. J., Macdonald, S., Evans, R. E., Marui, T. and Arai, S. Morpholine, bile acids and skin mucus as possible chemical cues in salmonid homing: electrophysiological re-evaluation. In: *Mechanisms of Migration in Fishes*, edited by J. D. McCleave, G. P. Arnold, J. D. Dodson and W. H. Neill. New York: Plenum Publishing Corporation, 1984, p. 363-378.

Harrison, T. A. and Scott, J. W. Olfactory bulb responses to odor stimulation: Analysis of response pattern and intensity relationships. *J. Neurophysiol.* 56:1571-1589, 1986.

Holley, A., Duchamp, A., Revial, M. F. and Juge, A. Qualitative and quantitative discrimination in the frog olfactory receptors: Analysis from electrophysiological data. *Ann. N. Y. Acad. of Sci.* 237:102-114, 1974.

Holley, A. Neural coding of olfactory information. In: *Smell and Taste in Health and Disease*, edited by T. V. Getchell. New York: Raven Press, 1991, p. 329-340.

Holley, A. and DØving, K. B. Receptor sensitivity, acceptor distribution, convergence and neural coding in the olfactory system. In: *Olfaction and taste VI*, edited by J. Le Magnen and P. Mac Leod. London: I.R.L., 1977, p. 113-123.

Hudson, W. W. Elementary techniques for assessing single-client\single-worker interventions. *Soc. Serv. Rev.* June:311-326, 1977.

Hyman, A. M. and Frank, M. E. Effects of binary taste stimuli on the neural activity of the hamster chorda tympani. *J. Gen. Physiol.* 76:125-142, 1980.

Imamura, K., Mataga, N. and Mori, K. Coding of odor molecules by mitral/tufted cells in rabbit olfactory bulb. I. Aliphatic compounds. *J. Neurophysiol.* 68:1986-2002, 1992.

Ivanova, T. T. and Caprio, J. Odorant receptor activated by amino acids in sensory neurons of the channel catfish Ictalurus punctatus. *J. Gen. Physiol.* 102:1085-1105, 1993.

Johannes, R. E. and Webb, K. L. Release of dissolved organic compounds by marine and fresh water invertebrates. In: *Symposium on Organic Matter in Natural Waters*, edited by D. W. Hood. Alaska: Alaska Institute of Marine Science, 1970, p. 257-274.

Johnson, B. R., Borroni, P. F. and Atema, J. Mixture effects in primary olfactory and gustatory receptor cells from the lobster. *Chem. Senses* 10:367-373, 1985.

Johnson, B. R., Voigt, R. and Atema, J. Response properties of lobster chemoreceptor cells: response modulation by stimulus mixtures. *Physiol. Zool.* 62:559-579, 1989.

Kalinoski, D. L., Bruch, R. C. and Brand, J. G. Differential interaction of lectins with chemosensory receptors. *Brain Res.* 418:34-40, 1987.

Kang, J. and Caprio, J. Electro-olfactogram and multiunit olfactory receptor responses to complex mixtures of amino acids in the channel catfish, Ictalurus punctatus. *J. Gen. Physiol.* 98:699-721, 1991.

Katoh, K., Koshimoto, H., Tani, A. and Mori, K. Coding of odor molecules by mitral/tufted cells in rabbit olfactory bulb. II. Aromatic compounds. *J. Neurophysiol.* 70:2161-2175, 1993.

Kauer, J. S. Response patterns of amphibian olfactory bulb neurons to odour stimulation. *J. Physiol.* 243:695-715, 1974.

Kauer, J. S. Coding in the olfactory system. In: *Neurobiology of Taste and Smell*, edited by T. E. Finger and W. L. Silver. New York: John Wiley & Sons, Inc., 1987, p. 205-231.

Kauer, J. S. Contributions of topography and parallel processing to odor coding in the vertebrate olfactory pathway. *TINS* 2:79-85, 1991.

Kauer, J. S. and Moulton, D. G. Responses of olfactory bulb neurons to odour stimulation of small nasal areas in the salamander. *J. Physiol.* 243:717-737, 1974.

Kauer, J. S. and Shepherd, G. M. Analysis of the onset phase of olfactory bulb unit responses to odour pulses in the salamander. *J. Physiol.* 272:495-516, 1977.

Kleerekoper, H. and Mogensen, J. A. The chemical composition of scent of fresh water fish with special reference to amines and amino acids. *Z. Vergl. Physiologie* 42:492-500, 1959.

Kleerekoper, H. and Mogensen, J. A. Role of olfaction in the orientation of petromyzon marinus. I. Response to a single amine in prey's body odor. *Physiol. Zool.* 4:347-360, 1963.

Kotrschal, K. Solitary Chemosensory Cells - taste, common chemical sense or what? *Rev. FishBio. Fisheries* 1:3-22, 1991.

Kroeze, J. H. A. After repetitious sucrose stimulation saltiness suppression in NaCl-sucrose mixtures is diminished: Implications for a central mixture suppression mechanism. *Chem. Senses* 7:81-92, 1982.

Laing, D. G. and Glemarec, A. Selective attention and the perceptual analysis of odor mixtures. *Physiol. Behav.* 52:1047-1053, 1992.

Laing, D. G. and Willcox, M. E. Perception of components in binary odour mixtures. *Chem. Senses* 7:249-264, 1983.

Lancet, D. The strong scent of success. *Nature* 351:275-276, 1991.

Lawless, H. T. Evidence for neural inhibition in bittersweet taste mixtures. *J. Comp. Physiol. Psychol.* 93:538-547, 1979.

Li, W and Sorensen, P. W. The olfactory sensitivity of sea lamprey is specifically restricted to arginine. *Chem. Senses* 17:658, 1992.(Abstract)

MacLeod, N. K. Spontaneous activity of single neurons in the olfactory bulb of the rainbow trout (Salmo gairdneri) and its modulation by olfactory stimulation with amino acids. *Exp. Brain Res.* 25:267-278, 1976.

Mair, R. G. Response properties of rat olfactory bulb neurons. *J. Physiol.* 326:341-359, 1982a.

Mair, R. G. Adaptation of rat olfactory bulb neurons. *J. Physiol.* 326:361-369, 1982b.

Marty, A. and Neher, E. Tight-seal whole-cell recording. In: *Single-channel Recording*, edited by B. Sakmann and E. Neher. New York, London: Plenum Press, 1983, p. 107-121.

Mathews, D. F. Response patterns of single neurons in the tortoise olfactory epithelium and olfactory bulb. *J. Gen. Physiol.* 60:166-180, 1972a.

Mathews, D. F. Response patterns of single units in the olfactory bulb of the rat to odor. *Brain Res.* 47:389-400, 1972b.

McClintock, T. S. and Ache, B. W. Hyperpolarizing receptor potentials in lobster olfactory receptor cells: implications for transduction and mixture suppression. *Chem. Senses* 14:637-647, 1989.

Meredith, M. The analysis of response similarity in single neurons of the goldfish olfactory bulb using amino-acids as odor stimuli. *Chem. Senses* 6:277-293, 1981.

Meredith, M. Patterned response to odor in mammalian olfactory bulb: the influence of intensity. *J. Neurophysiol.* 56:572-597, 1986.

Meredith, M. and Moulton, D. G. Patterned response to odor in single neurons of goldfish olfactory bulb: Influence of odor quality and other stimulus parameters. *J. Gen. Physiol.* 71:615-643, 1978.

Michel, W., Robinson, J. J.,II and Caprio, J. Olfactory and gustatory responses of the channel catfish, Ictalurus punctatus, to nucleotides. *Chem. Senses* 13:717, 1988. (Abstract)

Michel, W. C., McClintock, T. S. and Ache, B. W. Inhibition of lobster olfactory receptor cells by an odor-activated potassium conductance. *J. Neurophysiol.* 65:446-453, 1991.

Michel, W. C. and Ache, B. W. Odor-activated K^+ conductance inhibits lobster olfactory receptor cells. *Chem. Senses* 15:619-620, 1990.(Abstract)

Michel, W. C. and Ache, B. W. Odor-evoked inhibition in primary olfactory receptor neurons. *Chem. Senses* 19:11-24, 1994.

Miyamoto, T., Restrepo, D., Cragoe, E. J.,Jr. and Teeter, J. H. IP_3- and cAMP-induced responses in isolated olfactory receptor neurons from the channel catfish. *J. Membr. Biol.* 127:173-183, 1992a.

Miyamoto, T., Restrepo, D. and Teeter, J. H. Voltage-dependent and odorant-regulated currents in isolated olfactory receptor neurons of the channel catfish. *J. Gen. Physiol.* 99:505-530, 1992b.

Mori, K., Mataga, N. and Imamura, K. Differential specificities of single mitral cells in rabbit olfactory bulb for a homologous series of fatty acid odor molecules. *J. Neurophysiol.* 67:786-789, 1992.

Nevitt, G. A. and Moody, W. J. An electrophysiological characterization of ciliated olfactory receptor cells of the coho salmon Oncorhynchus kisutch. *J. Exp. Biol.* 166:1-17, 1992.

Ngai, J., Chess, A., Dowling, M. M., Necles, N., Macagno, E. R. and Axel, R. Coding of olfactory information: Topography of odorant receptor expression in the catfish olfactory epithelium. *Cell* 72:667-680, 1993a.

Ngai, J., Dowling, M. M., Buck, L., Axel, R. and Chess, A. The family of genes encoding odorant receptors in the channel catfish. *Cell* 72:657-666, 1993b.

Novoselov, V. I., Krapivinskaya, L. D. and Fesenko, E. E. Molecular mechanisms of odor sensing. V. Some biochemical characteristics of the alanineous receptor from the olfactory epithelium of the skate Dasyatis pastinaca. *Chem. Senses* 5:195-203, 1980.

O'Connell, R. J. and Mozell, M. M. Quantitative stimulation of frog olfactory receptors. *J. Neurophysiol.* 32:51-63, 1969.

Ohno, T., Yoshii, K. and Kurihara, K. Multiple receptor types for amino acids in the carp olfactory cells revealed by quantitative cross-adaption method. *Brain Res.* 310:13-21, 1984.

Potter, H. and Chorover, S. L. Response plasticity in hamster olfactory bulb: Peripheral and central processes. *Brain Res.* 116:417-429, 1976.

Rehnberg, B. G. and Schreck, C. B. The olfactory L-serine receptor in coho salmon: Biochemical specificity and behavioral response. *J. Comp. Physiol. [A]* 159:61-67, 1986.

Reinken, U. and Schmidt, U. Reactions of olfactory bulb neurons to different stimulus intensities in laboratory mice. *Exp. Brain Res.* 63:151-157, 1986.

Restrepo, D., Miyamoto, T., Bryant, B. P. and Teeter, J. H. Odor stimuli trigger influx of calcium into olfactory neurons of the channel catfish. *Science* 249:1167-1168, 1990.

Restrepo, D., Boekhoff, I. and Breer, H. Rapid kinetic measurements of second messenger formation in olfactory cilia from channel catfish. *Am. J. Physiol. Cell Physiol.* 264:C906-C911, 1993.

Restrepo, D. and Boyle, A. G. Stimulation of olfactory receptors alters regulation of $[Ca_i]$ in olfactory neurons of the catfish (Ictalurus punctatus). *J. Membr. Biol. 120:223-232, 1991.*

Restrepo, D. and Teeter, J. H. Olfactory neurons exhibit heterogeneity in depolarization-induced calcium changes. *Am. J. Physiol.* 258:C1051-C1061, 1990.

Revial, M. F., Duchamp, A. and Holley, A. Odour discrimination by frog olfactory receptors: A second study. *Chem. Senses* 3:7-21, 1978a.

Revial, M. F., Duchamp, A., Holley, A. and Mac Leod, P. Frog olfaction: Odour groups, acceptor distribution and receptor categories. *Chem. Senses Flav.* 3(1):23-33, 1978b.

Revial, M. F., Sicard, G., Duchamp, A. and Holley, A. New studies on odour discrimination in the frog's olfactory receptor cells. I. Experimental results. *Chem. Senses* 7:175-190, 1982.

Revial, M. F., Sicard, G., Duchamp, A. and Holley, A. New studies on odour discrimination in the frog's olfactory receptor cells. II. Mathematical analysis of electrophysiological responses. *Chem. Senses* 8:179-194, 1983.

Rhein, L. D. and Cagan, R. H. Biochemical studies of olfaction: binding specificity of odorants to cilia preparation from rainbow trout olfactory rosettes. *J. Neurochem.* 41:569-577, 1983.

Satou, M. Synaptic organization, local neuronal circuitry, and functional segregation of the teleost olfactory bulb. *Prog. Neurobiol.* 34:115-142, 1990.

Schild, D. Response pattern features of mitral cells in the goldfish olfactory bulb. *Brain Res.* 405:364-370, 1987.

Schild, D. and Zippel, H. P. The influence of repeated natural stimulation upon discharge patterns of mitral cells of the goldfish olfactory bulb. *J. Comp. Physiol. [A]* 158:563-571, 1986.

Scholz, A., Reid, G., Vogel, W. and Bostock, H. Ion channels in human axons. *J. Neurophysiol.* 70:1274-1279, 1993.

Shepherd, G. M. Olfactory bulb. In: *The Synaptic Organization of the Brain*, New York: Oxford University Press, 1979, p. 152-183.

Shibuya, T. and Shibuya, S. Olfactory epithelium: Unitary responses in the tortoise. *Science* 140:495-496, 1963.

Shibuya, T. and Tucker, D. Single unit responses of olfactory receptors in vultures. In: *Olfaction and Taste II*, edited by T. Hayashi. New York: Oxford Pergamon Press, 1967, p. 219-233.

Sicard, G. Olfactory discrimination of structurally related molecules: Receptor cell responses to camphoraceous odorants. *Brain Res.* 326:203-212, 1985.

Sicard, G. and Holley, A. Receptor cell responses to odorants: Similarities and differences among odorants. *Brain Res.* 292:283-296, 1984.

Silver, W. L., Caprio, J., Blackwell, J. F. and Tucker, D. The underwater electro-olfactogram: A tool for the study of the sense of smell of marine fishes. *Experientia* 32:1216-1217, 1976.

Silver, W. L. Olfactory responses from a marine elasmobranch, the Atlantic stingray, Dasyatis sabina. *Mar. Behav. Physiol.* 6:297-305, 1979.

Silver, W. L. Electrophysiological responses from the peripheral olfactory system of the American eel, Anguilla rostrata. *J. Comp. Physiol. [A]* 148:379-388, 1982.

Spath, M. and Schweickert, W. The effect of metacaine (MS-222) on the activity of the efferent and afferent nerves in the teleost lateral-line system. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 297:9-16, 1977.

Stabell, O. B. and Selset, R. Comparison of mucus collecting methods in fish olfaction. *Acta Physiol. Scand.* 108:91-96, 1980.

Stewart, W. B. and Scott, J. W. Anaesthetic-dependent field potential interactions in the olfactory bulb. *Brain Res.* 103:487-499, 1976.

Sun, X. J., Fonta, C. and Masson, C. Odour quality processing by bee antennal interneurons. *Chem. Senses* 18:355-377, 1993.

Sutterlin, A. M. and Sutterlin, N. Electrical responses of the olfactory epithelium of Atlantic salmon (Salmo salar). *J. Fish. Res. Bd. Can.* 28:565-572, 1971.

Suzuki, N. Effects of different ionic environments on the responses of single olfactory receptors in the lamprey. *Comp. Biochem. Physiol. [A]* 61:461-467, 1978.

Suzuki, N. Responses of olfactory receptor cells to electrical and chemical stimulation. In: *Chemoreception in Fishes*, edited by T. J. Hara. Amsterdam: Elsevier Scientific Publishing Company, 1982, p. 93-108.

Suzuki, N. and Tucker, D. Amino acids as olfactory stimuli in freshwater catfish, Ictalurus catus (Linn.). *Comp. Biochem. Physiol. [A]* 40:399-404, 1971.

Sveinsson, T. and Hara, T. J. Analysis of olfactory responses to amino acids in arctic char (Salvelinus alpinus) using a linear multiple-receptor model. *Comp. Biochem. Physiol. [A]* 97:279-287, 1990a.

Sveinsson, T. and Hara, T. J. Multiple olfactory receptors for amino acids in arctic char (Salvelinus alpinus) evidenced by cross-adaptation experiments. *Comp. Biochem. Physiol. [A]* 97:289-293, 1990b.

Tucker, D. Rapid decline of olfactory and gustatory receptor sensitivities of wild catfish (Ictaluridae) after capture. *J. Fish. Res. Bd. Can.* 30:1243-1245, 1973.

Uskova, Y. T. and Chaykovskaya, A. V. The amino acid content of mucus from the skin of various marine fishes. *Hydrobiologia* J7:79-80, 1971.

Valentincic, T., Wegert, S. and Caprio, J. Learned olfactory discrimination versus innate taste responses to amino acids in channel catfish (Ictalurus punctatus). *Physiol. Behav.* 55:865-873, 1994.

Wellis, D. P., Scott, J. W. and Harrison, T. A. Discrimination among odorants by single neurons of the rat olfactory bulb. *J. Neurophysiol.* 61:1161-1177, 1989.

Yaghmai, R. and Hazelbauer, G. L. Ligand occupancy mimicked by single residue substitutions in a receptor: transmembrance signaling induced by mutation. *Proc. Natl. Acad. Sci. USA* 89:7890-7894, 1992.

Zimmer-Faust, R. K., Tyre, J. E., Michel, W. and Case, J. F. Chemical mediation of appetitive feeding in a marine decapod crustacean: The importance of suppression and synergism. *Biol. Bull.* 167:339-353, 1984.

Zippel, H. P., Lago-Schaaf, T. and Caprio, J. Ciliated olfactory receptor neurons in goldfish (Carassius auratus) partially survive nerve axotomy, rapidly regenerate and respond to amino acids. *J. Comp. Physiol. [A]* 173:537-547, 1993.

Appendix: Letter of Permission

The Rockefeller
University Press
Journals Office
222 East 70th Street
New York, New York 10021
(212) XXXXXXX 327-8011

20 August 1993

Dear Dr. Kang:

We shall be glad to grant you permission for the reproduction of the material you referred to in your letter of 13 August 1993.*

Our only requirements are that you also obtain permission from the author(s) and give suitable acknowledgement to the source in the following manner: Reproduced from The Journal of General Physiology, year, vol., pp. by copyright permission of the Rockefeller University Press.

Yours truly,

THE JOURNAL OF GENERAL
PHYSIOLOGY

E Horak/mal
jc

Elizabeth Horak
Permissions

P.S. Since you are the author, a credit line is our only requirement.

Dr. Jiesheng Kang
Dept. of Zoology & Physiology
Louisiana State University
Baton Rouge, LA 70803

*JGP - 98:699-721, 1991 - article

VITA

Jiesheng Kang as born in Huian, Fujian, P.R.China on February 10, 1960. He received his high school diploma from the Fourth High School of Huian, Fujian, China in July, 1977. After working as an accountant for one year in a local farm, he entered Xiamen University in China majoring in Marine Biology in September, 1978. He received the Bachelor of Science degree in July, 1982. He then worked as a research and teaching assistant for five years and an instructor for one-and-a-half years in the Department of Oceanography, Xiamen University. In August, 1988, he came to LSU as a graduate student under Dr. John Caprio in the Department of Zoology and Physiology. He graduated with the Master of Science degree in Physiology in August, 1990. In August, 1990, he began to pursue his doctoral studies at LSU under the direction of Dr. John Caprio.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Jiesheng Kang

Major Field: Physiology

Title of Dissertation: Electrophysiological Responses of Olfactory Receptor and Bulb Neurons to Stimulus Mixtures and to Their Individual Components in the Channel Catfish, Ictalurus punctatus

Approved:

John T. Caprio
Major Professor and Chairman

Daniel Fogel
Dean of the Graduate School

EXAMINING COMMITTEE:

Kathryn A. Hamilt

Peter B. Johnson

George M. Shepherd

Thomas Dietz

J. Michael Hutchinson

Date of Examination:

May 13, 1994